Sugar transport in Trypanosoma brucei

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SUGAR TRANSPORT IN *TRYPANOSOMA BRUCEI*

Submitted by Stephen Game
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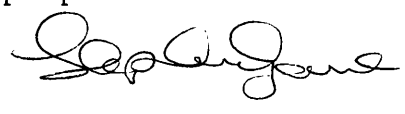

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Abbreviations

ADP - Adenosine diphosphate

ATP - Adenosine triphosphate

CNS - Central nervous system

DMF - Dimethyl formamide

EDTA - Ethylene diamine tetracetic acid

α-GP - α-glycerophosphate

GPO - Glycerol phosphate oxidase

KRP - Krebs-Ringer-Phosphate

NAD^+ - Nicotinamide adenine dinucleotide (oxidised form)

NADH - Nicotinamide adenine dinucleotide (reduced form)

P_i - inorganic phosphate

PTS - Phosphate transferase system

SHAM - Salicylhydroxamic acid

tBDSCl - tertiary-butyl-dimethyl-silyl chloride

THF - tetrahydrofuran

Summary

The transport of sugars by Trypanosoma brucei was investigated by measurement of influx and efflux of 6-deoxy-D-glucose, a non-metabolised D-glucose analogue. The necessary protocols required the organic synthesis of radio-labelled 6-deoxy-D-glucose for which suitable methods were developed.

Kinetic analysis of 6-deoxy-D-glucose transport indicated that the transport process is by facilitated diffusion and may be the rate-limiting step in the glycolytic pathway, the sole means of energy production in bloodstream form trypanosomes. More extensive kinetic analysis using zero-trans and equilibrium exchange procedures indicated that the hexose transporter is kinetically symmetrical.

The appropriate transport procedures were used to analyse the specificity requirements of the hexose binding site on the transport protein. The results indicated that the binding of hexose to the transporter differed significantly from other hexose transport systems including the rat adipocyte and the human erythrocyte. In particular, there appeared to be greater spatial freedom at the carbon-2 position compared with other mammalian hexose transporters. This could have important implications in the design of specific anti-trypanosomal agents.

The transport of 1-deoxy-D-glucose, a partially metabolised D-glucose analogue, indicated that the

phosphorylation of hexose may be closely linked to the transport process although phosphorylation was not a requirement for transport to occur.

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CHAPTER 5: DISCUSSION

CHAPTER 1: INTRODUCTION

1.1: The importance of trypanosomes

Trypanosomes are parasitic protozoa responsible for a variety of serious diseases in humans and their livestock throughout vast areas of the world. The African trypanosome possesses a number of unique features, the most important of which allows the organism to avoid the immune response of the host and defy any attempts to develop a vaccine. These unique features combined with the lack of effective disease treatment and control have stimulated considerable scientific interest into all aspects of the protozoan and the diseases it causes. The salivarian trypanosome Trypanosoma brucei brucei , the causative agent of the bovine disease 'nagana', is one of the most widely studied of any parasitic protozoa during the last 20 years. Trypanosoma brucei brucei is both morphologically and biochemically indistinguishable from the trypanosomes T. b. rhodesiense and T. brucei gambiense which,in man cause acute and chronic sleeping sickness respectively. A component of human serum thought to be high density lipoprotein (Rifkin, 1978) brings about the lysis of T. b. brucei but not T. b. rhodesiense or T. b. gambiense. Trypanosoma brucei brucei is, therefore, preferred for experimental research offering high yields of the bloodstream-form by infection of rodents with no risk of human infection.

The classification of the brucei subgroup of trypanosomes is shown in Figure 1. The genus trypanosoma

KINGDOM	:	ANIMALIA
SUB-KINGDOM	:	PROTOZOA
PHYLUM	:	SARCOMASTIGO PHORA
CLASS	:	ZOOMATIGOPHORA
ORDER	:	KINETOPLASTIDA
GENUS	:	TRYPANOSOMA
SECTION	:	SALIVARIA
SUB-GENUS	:	TRYPANOZOON
SPECIES	:	TRYPANOSOMA BRUCEI BRUCEI
		TRYPANOSOMA BRUCEI RHODESIENSE
		TRYPANOSOMA BRUCEI GAMBIENSE

Figure 1: The classification of Trypanosoma brucei (after Cox, 1982; Molyneux and Ashford, 1983).

is subdivided into salivarian (salivary) or stercorarian (faecal) trypanosomes according to the mode of transmission by the insect vector. The main disease caused by stercorarian type trypanosomes, Chagas' disease, is caused by Trypanosoma cruzi. The trypanosome is passed from the hind gut of the vector, usually in reduviid (triatomid) bug, to the human host via the faeces through abrasions on the skin or through the internal mucosa following ingestion. It is estimated that between 13 and 14 million people are actually infected in Central and South America. The disease is chronic in nature although some 10-20% of the patients may eventually die due to secondary infections and the eventual autoimmune phase of the disease (Boreham, 1979; Fairlamb, 1982). There is no satisfactory treatment for the disease although some drugs are used in spite of the complex administration of the treatment and high toxicity of the drugs (Gutteridge, 1985).

1.2: Principal diseases caused by the T. brucei subgroup

1.2.1: African sleeping sickness

The protozoa which cause African sleeping sickness are classified as salivarian as they develop in the proboscis or salivary glands of the insect vector, being transmitted by an innoculative method during feeding (Smyth, 1976).

The chronic form of the disease is caused by infection with T. b. gambiense primarily in riverine

areas of West Africa, (Figure 2) this being the preferred habitat of the Glossina palpalis group of tsetse flies (Figure 3) which carry T. b. gambiense. The more acute form of the disease is caused by infection with T. b. rhodesiense occurring mainly in the savannah of Africa (Figure 2). Transmission is mainly by the species G. morsitans, (Figure 3), which prefer open scrubland and wooded areas as a habitat.

If left untreated, the disease is invariably fatal, the time of death generally being related to the form of the disease. The chronic form usually leads to death after between nine months and three years (Donaldson, 1979), whereas the acute form can prove fatal within several months (Meshnick, 1984). Some 45 million people live in endemic areas of sleeping sickness of which about 10,000 new cases are reported annually (Goodwin, 1985). However, only five million of these people are under regular surveillance and the actual incidence of sleeping sickness is probably much higher. In endimic areas of chronic sleeping sickness, epidemics regularly break out and the death toll can reach hundreds of thousands annually. These epidemics often occur during periods of economic or political difficulties when normal control measures often break down, such as occurred recently in Uganda (Gashumba, 1981).

The pathology of the two forms of sleeping sickness is very similar and it is often difficult to distinguish the form of the disease in areas where both can occur. In general, pathological changes occur at the site of

Figure 2: The distribution and occurence of sleeping sickness in Africa. Solid dots indicate areas of T. b. rhodesiense endemicity. The open areas are T. b. gambiense foci which have a higher level of endemicity and from which epidemics arise (Molyneux and Ashford, 1983).

Figure 3: Distribution of the tsetse fly belt in Africa. Lined areas show the distribution of Glossina palpalis and dotted areas the distribution of Glossina morsitans, two of the major species of tsetse fly involved in the transmission of sleeping sickness (Molyneux and Ashford, 1983).

infection, in the bloodstream, in the extravascular spaces of the lymphatic and connective tissues and in certain visceral organs including the heart. In the later stages, the central nervous system (CNS), particularly the brain and spinal cord, become affected. In the acute form, death often occurs before advanced CNS involvement. The lymphatic glands are not normally affected and lesions of the heart are more frequent and severe (Donaldson, 1979). The actual means by which trypanosomiasis causes death is still uncertain (Markell and Voge, 1981), but it is known that the parasites have an immunosuppressive action often leading to secondary infections such as pneumonia, a frequent cause of death (Molyneux and Ashford, 1983).

The pathology of the disease manifests itself in man initially as an irregular intermittent fever. The patient complains of headaches, pains in the joints, loss of weight and pruritus. Oedema of the face and anaemia may also be present. In the early stages of CNS involvement insomnia may occur but as the disease advances, epilepsy, manical behaviour, somnolence and coma are typical (Molyneux and Ashford, 1983).

1.2.2: Nagana

Nagana, the disease caused by *T. b. brucei* used in this study is an extremely important disease in its own right affecting all the domestic animals raised by man. Up to 10 million square kilometres of Africa

are infected with tsetse fly. This land is capable of supporting 140 million head of cattle while only 20 million are actually grazed there (Molyneux and Ashford, 1983) and of these over three million die each year (Fairlamb, 1982). Extra cattle production would reduce dependence of some countries on imported meat, increase the nitrogenous content of the soil, make available more draught animals and raise the nutritional level of the population. The major diseases caused by trypanosomes are summarised in Table 1. It is clear that the impact of the diseases is very large in world terms and it can be understood why the World Health Organisation has targeted trypanosomiasis as one of the six most important tropical diseases for scientific study (Trigg, 1979).

1.3: The variable surface antigen (VSA)

The diagnosis of human trypanosomiasis is often made difficult by a characteristic fluctuating parasitaemia with trypanosomes often undetectable in the bloodstream (Figure 4). This cyclic parasitaemia is due to the ability of the trypanosomes of the *brucei* subgroup to undergo antigenic variation. The bloodstream forms of *T. brucei* are completely enveloped in a monomolecular glycoprotein coat 12-15nm thick (Vickerman, 1969), produced while the parasite occupies the salivary gland of the insect vector (Vickerman *et al.*, 1980). The majority of the trypanosomes possess a single variant of

SPECIES	VECTOR	RESEVOIR	HOST(S)	LOCATION	DISEASE
T. Cruzi	Triatomid bug	Armadillo	Man	S. and Central America	Chagas Disease
T. B. gambiense	Tsetse fly	Pig	Man	W. and Central Africa	Chronic African sleeping sickness
T. B. rhodesiense	Tsetse fly	Wild Game	Man	E. African Savannah	Acute African sleeping sickness
T. B. brucei	Tsetse fly	Wild Game	Domestic animals	African tsetse fly belt	Nagana
T. B. vivax	Biting flies	Wild Game	Cattle	African tsetse fly belt	Nagana
T. congolense	Tsetse fly	Wild Game	Cattle	African tsetse fly belt	Nagana
T. evensi	Tabanid fly	Mammals in wild	Horses, camels	N. Africa, Asia, Middle East	Surre
T. equiperdum	Venereal contact	-	Horses	Africa, S. Europe, Middle East	Dourine
T. equinum	Tabanid fly Vampire bat	Mammals	Horses, Dogs, Cattle	S. America	Mal de Caderas

Table 1: Table of the main disease carrying trypanosomes (Hoare, 1964; Markell and Voge, 1981).

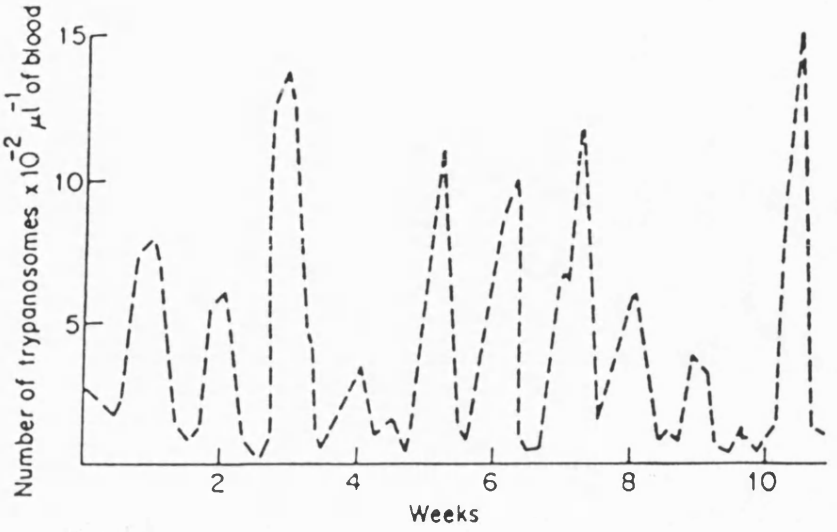


Figure 4: The fluctuating parasitaemia in the blood of a patient with African sleeping sickness (Wakelin, 1984).

the surface antigen, the homotype, but a small minority possess heterotypical surface antigens. After infection, the hosts immune system quickly responds by raising antibodies to the homotype and the trypanosomes are destroyed by agglutination and complement-mediated lysis (Cox, 1982; Polter, 1985; Vickerman, 1985). The heterotypical VSA's are not destroyed and the most common heterotype becomes the new homotype (Figure 5).

It has been estimated that a single trypanosome possesses up to 1000 genes encoding for the VSA representing between 5 and 10% of the total genetic capacity of the organism (Donelson and Turner, 1985). Recently the genetics and structure of the VSA have been extensively studied in the hope of eventually developing a vaccine against sleeping sickness. The VSA is a glycoprotein with a molecular weight of approximately 65,000 (Cross and Johnson, 1976) consisting of a single polypeptide chain of approximately 600 amino acid residues (Mansfield, 1981). The outer N-terminal domain is highly variable in amino acid sequence (Whitfield, 1979). There is thought to be a more conserved area of amino acid sequence in regions concerned with attachment and three dimensional conformation (Cross and Johnson, 1976). The attachment of the molecule to the membrane is *via* covalent linkage of the carboxy-terminal of the polypeptide to glycosyl-*sn*-1,2-dimistryl-phosphatidylinositol in the trypanosomal membrane, and the release of the coat is catalysed by an endogeneous phospholipase C (Ferguson *et al.*, 1985). To

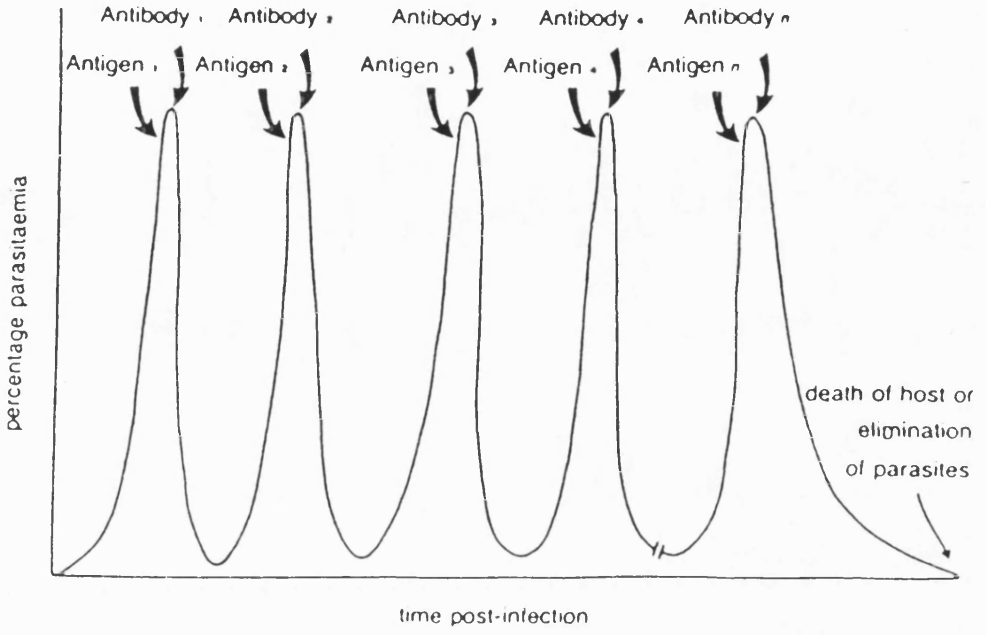


Figure 5: The role of antigenic variation in relapsing infection. The rising parasitaemia consists primarily of long slender forms, and the falling parasitaemia, of short stumpy forms (Chappell, 1980).

date there has been little success in the attempts to develop a vaccine. Currently the only possibility for vaccination against African trypanosomiasis seems to be against the limited number of VSA's which are expressed at the beginning of an infection (Donelson and Turner, 1985), although it is suggested that should these antigens be suppressed, another set would replace them (Kolata, 1984).

1.4: Control of African trypanosomiasis

The control of African trypanosomiasis centres on the avoidance of contact with the tsetse fly. This can be achieved by either moving human populations to tsetse fly-free areas or by reducing the man-fly contact by control of the size and distribution of the tsetse fly population. The former method is expensive, disruptive and not usually very effective as any success is quickly reversed by the breakdown of surveillance and diagnostic facilities which often occur in the African countries most at risk (Goodwin, 1985). The latter method depends on a variety of techniques including alteration of the habitat, pesticide application, genetic and biological control and physiological interference. Alteration of the habitat by clearing of the bush or the shooting of game animals is unacceptable on the grounds of conservation and is no longer in common use (Ukoli, 1984). Genetic control by the release of large numbers of sterilized male flies (Jordan, 1985) is expensive and has not been successful in eradicating the screw worm (Cochliomylia

hominivorax) in Northern Mexico where the influx of new flies from Southern Mexico has made complete control impossible. Similar difficulties are easy to envisage in Africa (Molyneux, 1982). Biological control by means of vector parasites or predators (Jordan, 1985) and physiological interference by means of insect growth regulators which inhibit chitin synthesis (Molyneux, 1982) are both subjects of continuing research. The use of insecticides by air or ground spraying is still widely practised in some areas of Africa but its limited effectiveness against sleeping sickness and the ecological and environmental impact restrict its suitability for more widespread use. The use of traps which attract tsetse flies both on a visual and with much more success, on an olfactory basis have been the subject of much interest recently. Synthetic organic compounds originally identified in ox breath have been successful in attracting tsetse flies to traps impregnated with insecticide. It is thought that suitable traps can reduce Glossina populations by over 99% in riverine areas (Laveissiere and Couret, 1980). However, sufficient numbers of traps together with the materials and manpower to maintain them effectively would still represent considerable expense to the African countries concerned.

The incidence of nagana could be reduced by the exploitation of trypanotolerence shown by some breeds of cattle. These cattle, primarily humpless taurine breeds, are smaller and less prolific milk producers

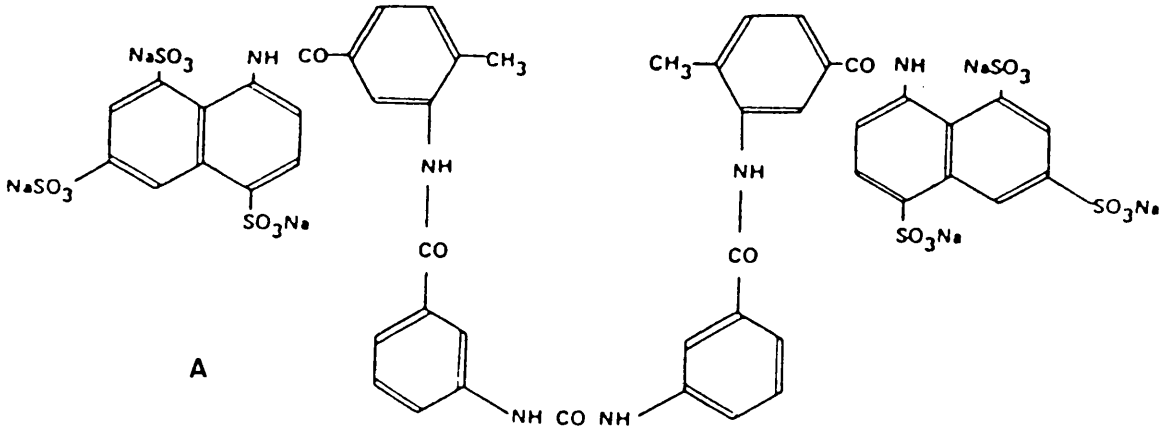
than the more commonly kept zebu. It remains for long term breeding programmes to combine the most desirable characteristics of various breeds (Molyneux and Ashford, 1983).

1.5: Anti-trypanosomal drugs

There are three main drugs currently in use for the treatment of African sleeping sickness. These drugs, all introduced before 1955 (Howells, 1985), are pentamidine for chemoprophylaxis, suramin for treatment of the early stages of the disease and melarsoprol for the later stages where CNS involvement occurs (Figure 6).

Pentamidine, a positively-charged aromatic diamidine, is the preferred agent for the prophylaxis of sleeping sickness and can also be used therapeutically against the early stages of the chronic and to a lesser extent the acute form of the disease. It cannot cross the blood-brain barrier and is, therefore, ineffective against the later stages when there is CNS involvement.

The selectivity of the drug for the parasite is due to the presence of a high-affinity energy-requiring transport system (Damper and Patton, 1976) which accumulates the drug in the parasite. The drug appears to exert its effect on the synthesis of deoxyribonucleic acid (DNA) with the synthesis of the uniquely trypanosomal kinetoplast DNA appearing to be particularly sensitive to disruption (Gutteridge, 1985). Side effects of pentamidine treatment are nephrotoxicity (Meshnik, 1985). Some parasite resistance to pentamidine is now becoming



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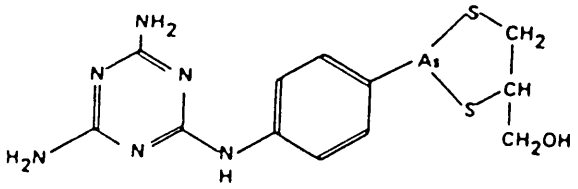

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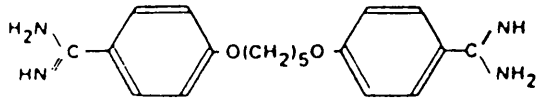

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Figure 6: The structures of the antitrypanosomal drugs most commonly used in the treatment of African sleeping sickness. A = suramin, B = melarsoprol, C = pentamidine. (Gutteridge, 1985).

apparent (Ree, 1985).

The sulphated-napthylamine suramin is also unable to penetrate the blood-brain barrier (Meshnik, 1984). The drug is administered to the patient intravenously and slowly taken up by endocytosis (Gutteridge, 1985). The drug works by inhibiting the enzymes involved in the reoxidation of NADH by the so-called α-glycerophosphate shuttle (Figure 9) thus reducing the rate of glycolysis and respiration (Fairlamb and Bowman, 1980b). One of these enzymes, α-glycerophosphate oxidase is found only in the trypanosome. The other, NAD^+-linked α-glycerophosphate dehydrogenase is found in both trypanosome and mammalian cells (Gutteridge, 1985). The selectivity of suramin is, therefore, not reliable and produces a range of serious side-effects varying from immediate nausea and collapse to blindness and renal failure, often fatal. The third drug melarsoprol, an aromatic arsenical can rapidly cure all stages of both chronic and acute African sleeping sickness. In cell-free homogenates, melarsoprol has been shown to inhibit several enzymes of carbohydrate metabolism including pyruvate kinase (Flynn and Bowman, 1974), glycerol kinase (Hammond and Bowman, 1980b) and α-glycerophosphate oxidase (Fairlamb and Bowman, 1977). Trivalent arsenicals have a high affinity for sulphydryl groups which are present in the active sites of many enzymes, particularly kinases. Flynn and Bowman (1974) have shown that pyruvate kinase is the enzyme most affected *in vivo* as it is located in the cytoplasm of the cell. The

other enzymes mentioned are all present in a microsome-like organelle, the glycosome (Fairlamb, 1982). The slight degree of preferential binding to trypanosomal pyruvate kinase over host pyruvate kinase gives some small degree of selectivity for melarsoprol (Gutteridge, 1985). There are however, serious side effects. Up to 18% of patients suffer reactive encephalopathy which occasionally proves fatal and more commonly fever and pain in the chest and abdomen. Melarsoprol is usually only administered under close hospital supervision (Molyneux and Ashford, 1983).

It is clear from the description of the most widely used drugs that further research into anti-trypanosomal drugs is necessary. Current activity has moved away from the random screening of potential drugs to directing agents against unique aspects of trypanosomal metabolism and morphology. In this respect the current research into the biochemistry and molecular biology of trypanosomes provides valuable information which could lead to the development of an important new drug or vaccine.

1.6: The morphology and life cycle of T. brucei

There are several unique features of the morphology of the trypanosome which have been the subject of much recent research. The ultrastructure of a typical bloodstream form of trypanosome is shown in Figure 7. One feature, the protein coat which can be shed to vary the surface antigen, has been described in Section 1.3.

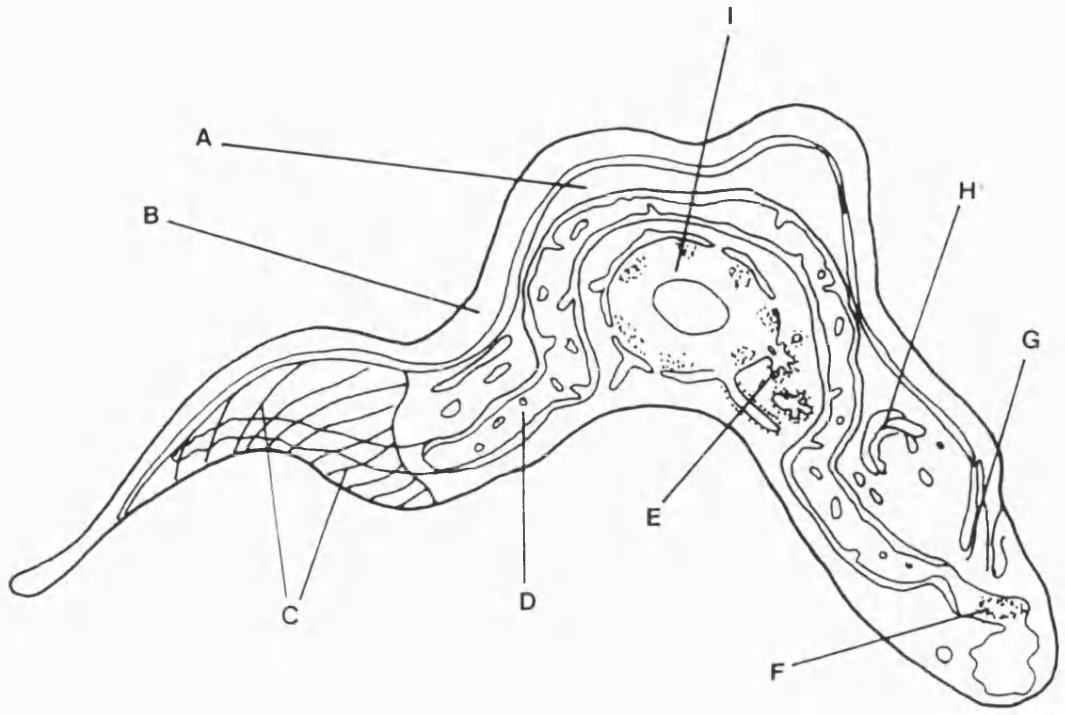


Figure 7: The ultrastructure of a bloodstream form of trypanosome. Long slender forms are approximately 30μm in length. Short stumpy forms are between 14 and 20μm in length. A - fold of pellicle. B - attached flagellum. C - microtubules of pellicle (anterior end only). D - mitochondrion. E - endoplasmic reticulum. F - kinetoplast. G - basal body of flagellum. H - golgi apparatus. I - nucleus.

Beneath the plasma membrane a regular array of pellicular microtubules forms a cytoskeleton in both the vertebrate and invertebrate forms of the parasite (Molyneux and Ashford, 1983).

The kinetoplast located at the basal end of the mitochondrion is a complex network of mitochondrial DNA representing 30% of the total cellular DNA (Opperdoes, 1985). One component of the kDNA network, the maxicircle, has features similar to mitochondrial DNA. The major transcripts are ribosomal and messenger RNA molecules (Fairlamb, 1982) and there is also evidence that the genes for cytochrome oxidase subunits I, II, and III and apocytochrome b are present within the maxicircles. The second component of the kinetoplast, the minicircles, of which there are approximately 6000, has a heterogeneous nucleotide sequence but as yet no known function (Opperdoes, 1985).

The trypanosome also contains a microsome-like organelle, termed the glycosome by Opperdoes and Borst (1977), containing the major enzymes of the glycolytic pathway, adenylate kinase and enzymes of the pyrimidine biosynthetic pathway (Opperdoes and Cottem, 1982). A single trypanosome contains an average of 230 glycosomes representing approximately 4.3% of the total cell volume. Each glycosome is spherical or eliptical and surrounded by a single membrane (Opperdoes et al., 1984).

The single mitochondrion present in trypanosomes undergoes striking changes during the various stages of

the lifecycle. In the insect stages, the mitochondrion is fully developed with numerous plate-like cristae, while the mammalian forms have a simple unbranched mitochondrion with few tubular cristae (Fairlamb, 1982). It is the development of the mitochondrion combined with the change in shape of the organism, the position of the flagellum (Hoare and Wallace, 1966) and the position of the kinetoplast relative to the nucleus (Vickerman, 1985) which describe the morphological stages of the life cycle (Figure 8).

The form injected by the infected tsetse fly into the host is the metacyclic form which rapidly transforms into the trypomastigote form which multiplies to form a characteristic chancre. The trypomastigotes migrate to the lymph nodes and then to the bloodstream. In a typical chronic relapsing infection, bloodstream forms display a heterogeneity of morphology known as pleomorphism (Markell and Voge, 1981). The long slender form is more numerous in the rising parasitaemia while the short stumpy form is usually seen in the remission stage. This is thought to be a preadaption for the insect stage of the life-cycle (Molyneux and Ashford, 1985). After the tsetse fly has fed on the infected host the short stumpy forms ingested transform in the insect midgut to procyclic forms which multiply, then migrate to the salivary glands. In the salivary glands the procyclic form transforms first into the epimastigote then once again into the infective metacyclic form

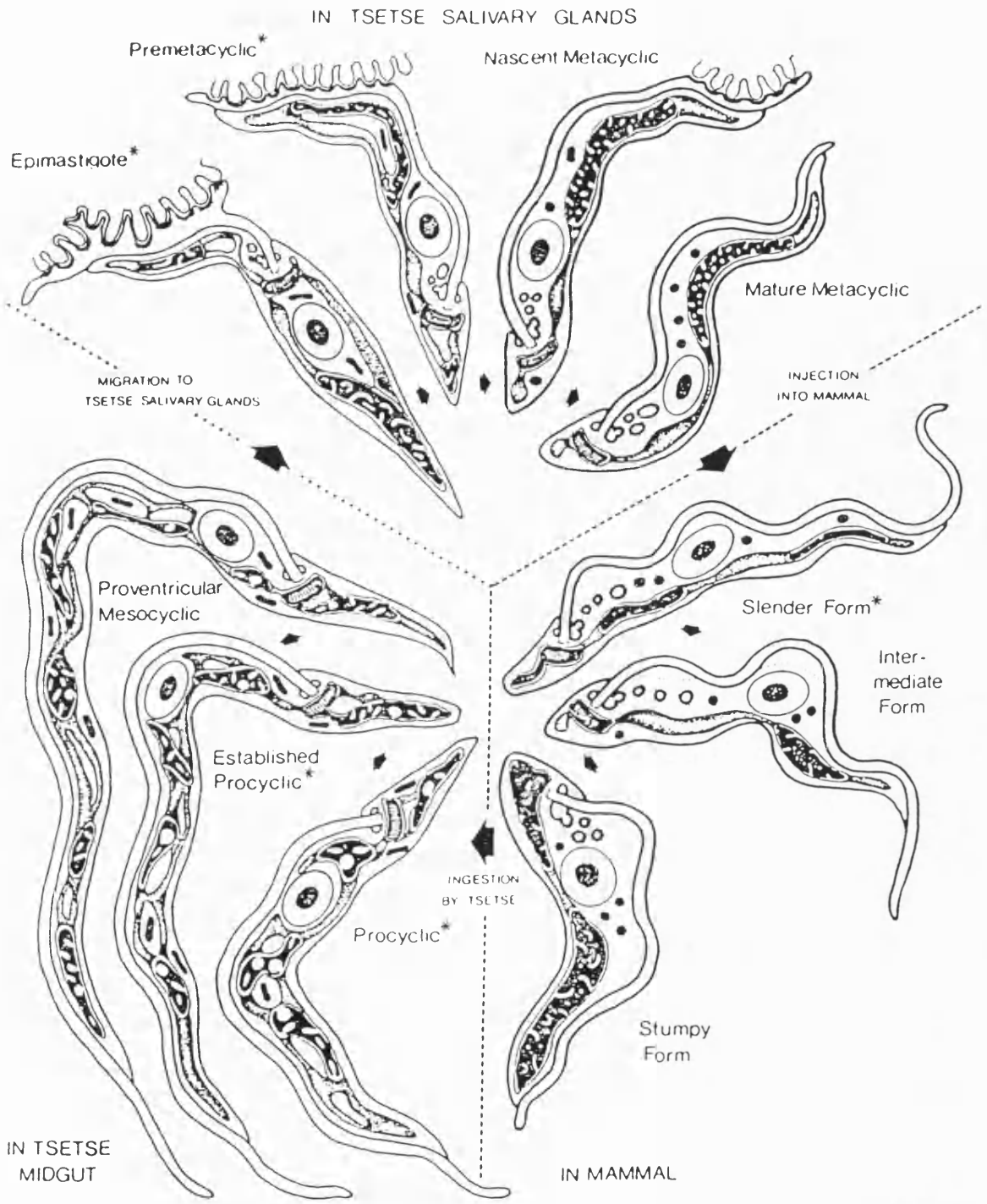


Figure 8: The developmental cycle of Trypanosoma brucei in the mammalian host and the tsetse vector showing the relative sizes of the various stages. The forms marked with an asterisk are those in which division occurs. The forms marked with thick outer membranes are those with a protein coat (Vickermann, 1985).

(Vickerman, 1985). Multiplication is believed to be by binary fission (Cox, 1982), however, the possibility of a sexual cycle in members of the brucei subgroup has been suggested by recent workers (Jenni et al., 1986: Vickerman, 1986).

The changes in morphology during the life-cycle are associated with the ability to adapt the respiratory pathway to the prevailing conditions. The principle energy source in the insect stage is proline, the amino acid most abundant in the haemocoel of the tsetse fly (Srivastava and Bowman, 1971) and the transformation from mammalian to insect form is accompanied by a marked increase in proline oxidase activity (Brown et al., 1973). Proline is present at concentrations up to 150mM, being the source of energy for insect flight (Bursell, 1966).

The transition from mammalian to procyclic insect form via the short stumpy form is accompanied by synthesis of certain enzymes of the tricarboxylic acid (TCA) cycle, α-oxglutarate oxidase (Flynn and Bowman, 1973) for example. However, low levels of citrate synthase and succinate dehydrogenase seen in short stumpy forms have led to the conclusion that the TCA cycle is not fully functional in vivo (Flynn and Bowman, 1973). The major end products of aerobic respiration in procyclic forms of T. brucei are succinate, malate, fumarate and aniline (Bowman and Flynn, 1976).

The insect forms of the brucei subgroup have at least three terminal respiratory systems. Of these, one has cyanide-sensitive cytochrome a-a-3 as its terminal oxidase, one has salicylhydroxamic acid (SHAM)-sensitive -α-glycerophosphate oxidase and the third, sensitive to neither SHAM or cyanide is as yet unknown but has been suggested as cytochrome-o (Njogu et al., 1980). The former two are the most important, contributing 60% and 30% respectively to the total cellular respiration (Njogu et al., 1980).

1.7: Energy metabolism in bloodstream-forms of T. brucei

Energy production in the bloodstream-form of T. brucei is markedly different from those forms which inhabit the insect. The long-slender bloodstream form, as used throughout this work, possesses a simple mitochondrion, an incomplete TCA cycle and no functional respiratory chain (Fairlamb, 1982). Bloodstream trypomastigotes contain little if any storage carbohydrate (Opperdoes et al., 1976b) and are, therefore, reliant on glycolysis for the production of energy. The absence of external substrate results in rapid depletion of ATP levels (Opperdoes et al., 1976). Bloodstream trypanosomes respire actively on only four substrates: D-glucose, D-fructose, D-Mannose and glycerol (Ryley, 1956; 1962). In addition, short stumpy trypomastigotes will respire with α-oxogluterate at about half the rate found with D-glucose (Flynn and Bowman, 1973). The

human bloodstream contains D-glucose at a concentration of approximately 5mM (Whitfield, 1979), this constant supply eliminating the need for energy reserves in the parasite. Respiratory activity is correspondingly very high, oxygen consumption at 37°C being 120 - 180 nmol/min/mg protein, compared to 1 - 10 nmol/min/mg protein for mammalian cells (Bowman and Flynn, 1976).

It may be to maintain this high rate of glycolysis that the major part of the glycolytic pathway is contained in the glycosome (Figure 9). As a consequence of the incomplete and non-functional TCA cycle and lack of lactate dehydrogenase in the bloodstream form (Bird et al., 1971), glucose is metabolised almost exclusively to pyruvate under aerobic conditions, this being excreted into the bloodstream of the host (Ryley, 1956; Flynn and Bowman, 1973). The reduced NADH produced during the conversion of a triose phosphate to pyruvate is reoxidised by means of the α-glycerophosphate oxidase (GPO) shuttle shown in Figure 9, located in the mitochondrion (Opperdoes et al., 1977). This terminal respiratory system is not sensitive to typical inhibitors of the mammalian respiratory chain, cyanide for example, and it is generally accepted that there are no functional cytochromes present in the bloodstream forms of organisms of the Trypanozoon subgenus (Ryley, 1956; Grant and Sargent, 1961; Ryley, 1962). It can be seen from Figure 9 that the net production of ATP during aerobic respiration is 2 molecules, sufficient to maintain cellular function

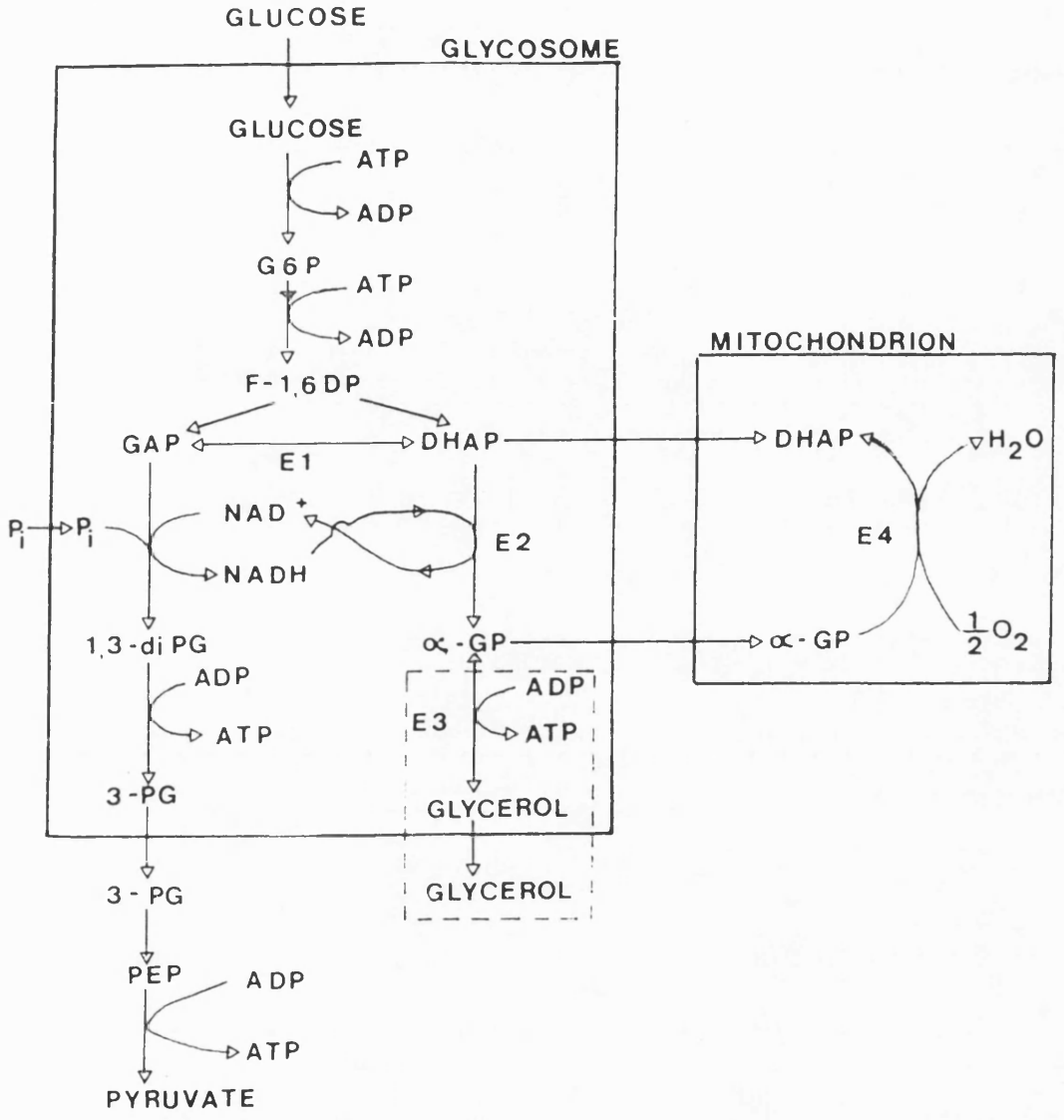


Figure 9: Pathways of glycolysis in bloodstream form trypanosomes. The reactions enclosed in the broken box are assumed present under anaerobic conditions. The enzymes indicated are E1 - triosephosphate isomerase, E2 -α glycero-phosphate dehydrogenase, E3 - glycerol kinase, E4 - α-glycerophosphate oxidase. Other abbreviations: G6P - glucose 6 phosphate, F-1,6DP - fructose 1,6 diphosphate, GAP - glyceraldehyde-3-phosphate, 1,3-diPG - 1,3 diphosphoglycerate, 3-PG - 3 phosphoglycerate and PEP - phosphoenol pyruvate.

(Brohn and Clarkson, 1980).

Under anaerobic conditions, the GPO shuttle is inoperative. D-glucose is metabolised to approximately equimolar concentrations of pyruvate and glycerol (Opperdoes et al., 1976b). Thus the redox balance of the trypanosomes is maintained (Bowman and Flynn, 1976). Since bloodstream forms are able to survive anaerobiosis there must be a net synthesis of ATP. Several hypothetical schemes for the production of net ATP have been proposed (Clarkson and Brohn, 1976), but that which has most evidence to support was put forward by Opperdoes and Borst (1977) who suggest that glycerol and ATP are synthesised from α-glycerophosphate (α-GP) and ADP by glycerol kinase. During anaerobiosis the intracellular concentration of the glycolytic intermediate α-GP rises by up to 30-fold (Hammond and Bowman, 1980a), with possible intraglycosomal concentrations of 0.1M being reached (Opperdoes and Borst, 1977). Although the reaction described is thermodynamically unfavourable (Mackenzie et al., 1983) the reaction may proceed as a result of the high local concentration of α-GP. The overall thermodynamics for the conversion of D-glucose to pyruvate and glycerol are however favourable and the necessity for an accumulation of α-GP has recently been questioned (Hammond et al., 1985).

Recent work on the aerobic/anaerobic transition in T. brucei has shown that at physiological oxygen concentrations, the anaerobic pathway is almost inoperative (Eisenthal and Panes, 1985) so the function of anaerobic

metabolism in bloodstream trypanosomes is largely unknown.

1.8: Substrate transport in trypanosomes

The bloodstream form of T. brucei is distinguished by having a very high metabolic rate (Bowman and Flynn, 1976) coupled to the generation of metabolic energy solely by glycolysis. There has been considerable scientific interest in the glycolytic pathway as a potential site for action of anti-trypanosomal agents but relatively little attention has been paid to the transport of metabolic substrates. Work carried out on T. Lewisi (Sanchez and Read, 1969) measured uptake of radio-labelled substrates into trypansomes incubated at 37°C. This involved the addition of radio-label to preincubated cells followed by the addition of 4 volumes of cold buffer to stop uptake after 10 minutes. The cells were then processed over a 30 minute period. The results indicated that D-glucose uptake was saturable (Km = 1.25mM) and on the evidence of inhibitor studies, it was suggested that two sites of carbohydrate transport existed. The first was preferentially used by D-glucose, and the second site by D-glucosamine, D-fructose, D-galactose and 3-0-methyl-D-glucose. Similar results were shown in T. b. gambiense (Southworth and Read, 1969; 1970) using similar assay procedures to Sanchez and Read, (1969). Inhibition studies again indicated two uptake sites, one used by D-glucose, D-mannose, glycerol and 2-deoxy-D-glucose, the other by D-fructose and D-glucosamine.

D-Galactose and 3-O-methyl-D-glucose did not inhibit uptake of glucose. Results from specificity studies using over 40 inhibitors in T. equiperdum (Ruff and Read, 1974) suggests the uptake system to be even more complex. Three sites of substrate uptake were proposed: a 'sugar' site through which D-glucose, D-fructose and D-mannose are transported, a 'glycerol' site through which the substrates described above may be transported and a 'glycerol' site for uptake of glycerol alone. Gruenberg et al., (1978) conducted a more detailed study of D-glucose transport in T. brucei. Their findings indicated that glucose is transported by an energy-independent carrier-mediated process and the permeation process is the rate-limiting step for the metabolism of D-glucose. Also, glycerol inhibition of hexose uptake was at the metabolic level rather than the carrier level. Whilst searching for a non-metabolised D-glucose analogue they found that 6-deoxy-D-glucose did not inhibit D-glucose uptake and 3-O-methyl-D-glucose did not follow Michaelis-Menten kinetics even at low concentrations.

All the work on substrate transport described followed the uptake of fully metabolised substrates or partially metabolised sugar analogues. Also, all measurements were under steady-state conditions rather than using initial rates. Under such conditions and with the extremely high metabolic rate of trypanosomes it is clear that interpretation of results solely in terms of the transport process is very difficult. This

study was therefore initiated to attempt to investigate the kinetics and specificity of the transport process by the use of suitable uptake procedures using non-metabolisable sugar analogues.

1.9: Types of transport systems

The processes by which small molecules or ions traverse the plasma membrane can be generally classified as follows (Figure 10):

a) Simple diffusion: Molecules diffuse across the membrane down their concentration gradients without the participation of specific carriers.

b) Facilitated diffusion: Molecules are conducted across the membrane down their concentration gradient by stereospecific protein carriers. There is no energy-coupling so the substrate is not concentrated within the cell. As transport is catalysed by a finite number of specific carriers, facilitated diffusion shows saturation kinetics and can be inhibited by structural analogues of the transport substrate.

c) Active transport: Substrates are transported by specific carriers but intracellular substrate concentrations may reach levels several hundred times that of the external medium. This accumulation against a concentration gradient requires the expenditure of energy, therefore active transport systems are also sensitive to

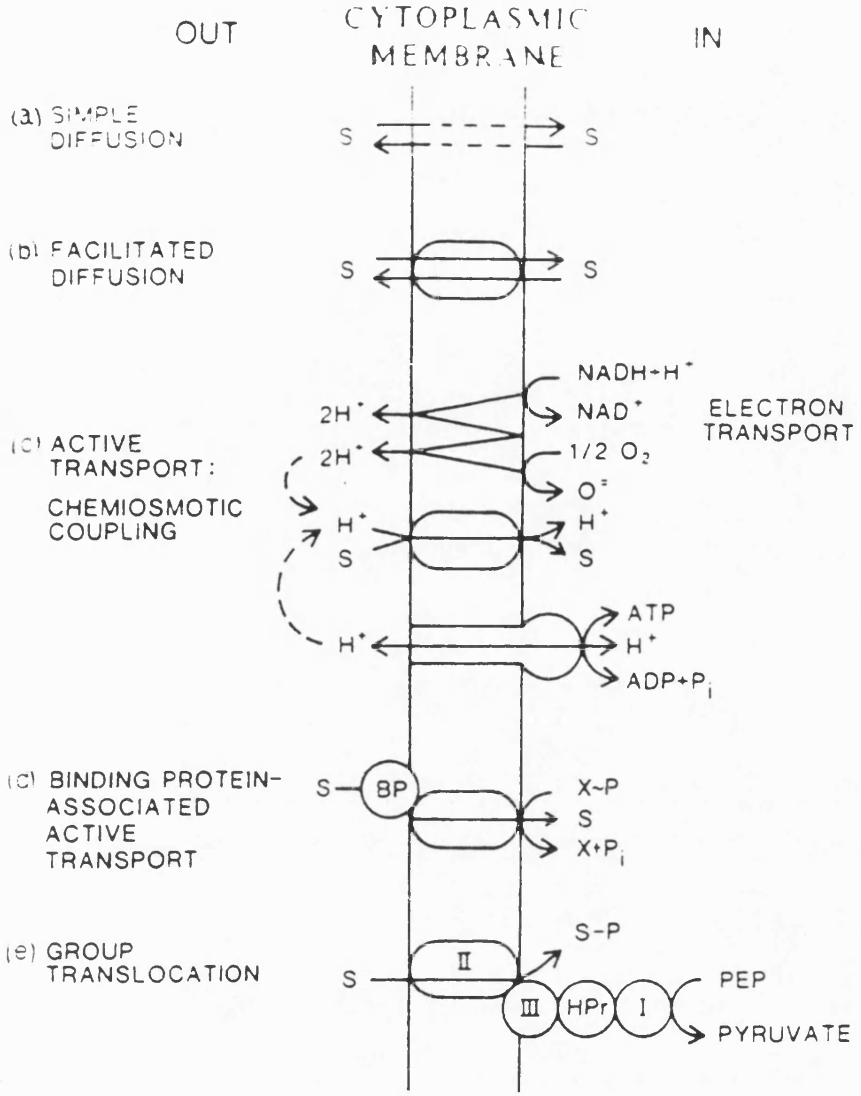


Figure 10: Types of transport systems found in microorganisms (from Romano, 1986). Abbreviations: S, substrate; S-P, phosphorylated substrate; Pi, inorganic phosphate; X~P, high energy phosphate compound; PEP, phosphoenol pyruvate; I, II, III, group translocase enzymes I, II, III; HPr, heat stable phosphocarrier protein; BP, binding protein.

metabolic inhibitors. There are two main classes of active transport mechanisms based on the means of coupling to metabolic energy:

i) Chemiosmotic coupling: In this system metabolic energy is expended to remove ions from the cell so that an inwardly directing electrochemical gradient is created. The substrate is then transported along with the ion into the cell *via* a 'symport' and can accumulate as long as the electrochemical gradient is maintained. An example of such a system is the sodium-dependent D-glucose transport system in mammalian intestine and kidney tubules (Crane, 1960).

ii) Direct chemical energy coupling: This system transports substrates actively, energized directly by phosphate bond energy. In addition to a membrane-bound permease, a binding protein is also required for transport. This binding protein requires a distinct periplasmic space between the cytoplasmic membrane and the outer membrane and such systems are therefore only found in Gram-negative bacteria (Silhavy *et al.*, 1978). The energetics of such systems are not well understood, but ATP is thought to be the probable energy source.

d) Group translocation: In this case substrates are chemically altered during the transport

process. The best known group translocation system is the phosphoenolpyruvate- sugar phosphotransferase system (PTS) that is widely distributed in bacteria (Kundig et al., 1964). The PTS is a multicomponent system consisting of a number of proteins that participate in the transfer of a phosphate group from phosphoenol-pyruvate to a number of sugars as they traverse the plasma membrane. As many as 12 different sugars can be transported by a given organism via the PTS. The sugar first appears in the cell as the phosphorylated derivative. The system plays a central role as a metabolic regulatory system. As phosphoenolpyruvate is a product of glycolysis, sugars can be accumulated without expense of ATP.

The evidence from previous work on sugar transport in T. brucei (Gruenberg et al., 1978) indicates that a facilitated diffusion system is operative for D-glucose uptake into trypanosomes.

1.10: Kinetic approaches to the study of sugar transport

The combination of a transported hexose with a membrane protein to form a transporter-substrate complex leads to saturation kinetics. This system differs from the familiar model of an enzymic reaction with one free enzyme form E (Scheme 1a) in that two separate binding sites (E_1 and E_2) are available for the substrate (S), one on each face of the membrane. Scheme 1b shows the

most widely-used model for facilitated diffusion, the carrier model. The subscripts 1 and 2 refer to the two sides of the membrane. Transport is measured as the movement of substrate from side 1 to side 2 or side 2 to side 1.

$$E + S \rightleftharpoons ES \longrightarrow P + E$$

Scheme 1a

$$\begin{array}{ccc} E_1 & \rightleftharpoons & E_2 \\ \uparrow\downarrow & & \uparrow\downarrow \\ S_1 & & S_2 \\ \uparrow\downarrow & & \uparrow\downarrow \\ ES_1 & \rightleftharpoons & ES_2 \end{array}$$

Scheme 1b

Kinetic analysis of the carrier model for facilitated diffusion will thus show half saturation constants (K_m's) and maximum velocities (V_{max}'s) which have different interpretations depending on the direction of flux and the substrate concentrations on each side of the membrane. Eilam and Stein (1974) showed that for any of the following protocol descriptions the rate of flux (V) will be given by an equation of the Michaelis-Menten form:

$$V = \frac{V_{max}S}{K_m + S} \qquad (1)$$

Kinetic constants for a facilitated diffusion system are analogous but not identical with those determined for an enzymic reaction.

A number of different experimental protocols are available to study the kinetics of facilitated diffusion.

The main technique for measuring transport rates is to follow the flux of a non-metabolised radio-labelled substrate from one face of the membrane (the cis face) to the opposite face (the trans face). The cis to trans flux of the substrate can be measured either into (entry or influx) or out of (exit or efflux) the cell. In all experiments unidirectional flux of the radio-labelled substrate is followed. This can only be achieved by measuring initial rates either at very short uptake times (since backflux of radio-labelled substrate will occur at longer uptake times) or by the use of suitable integrated rate equations to compensate for the backflux which occurs at longer times.

1.10.1: Equilibrium exchange experiments

In these experiments the initial rates of unidirectional flux of radio-labelled substrate are measured when there is no concentration gradient across the membrane i.e., the substrate concentration in the cis solution is equal to that in the trans solution. Under these conditions there is no net flux of substrate and the rates of unidirectional efflux and influx are the same. The entry curve is nearly exponential and follows with close approximation the equation:

$$A_t = A_\infty (1 - e^{-kt}) \qquad (2)$$

Where A_t and A_∞ denote the amount of intracellular radioactivity at time t and infinite time respectively.

The rate constant (k) is the transport velocity (V) divided by the substrate concentration (S). Thus equation 2 becomes:

$$K = V/S = \frac{\ln 1/(1-f_i)}{t} \qquad (3)$$

where f_i (fraction inside) = A_t/A_∞.

Therefore for equilibrium exchange experiments plots of V/S vs t should be linear at any given substrate concentration.

Once uptake has been shown to be exponential, equation 3 can be used to calculate the initial uptake rates. The value of V/S at different substrate concentrations can be plotted using the transformation of equation 1. For example the S/V vs S plot (Hanes' plot) transforms equation 1 into:

$$\frac{S}{V} = \frac{K_m}{V_{max}} + \frac{S}{V_{max}} \qquad (4)$$

The equilibrium exchange V_{max} and K_m reflect the properties of both the internal and external sites of the transport system when substrate is being transported in both directions.

1.10.2: Zero-trans experiments

In these experiments the initial rate of flux of substrate from cis to trans is followed when there is initially no substrate in the trans solution. Thus for a zero-trans entry experiment, substrate and radio-

labelled substrate are added to the external solution and the uptake is measured. The substrate enters the cell rapidly and therefore, over practical time courses, the substrate concentration will rise in the trans solution and backflux will occur. It is therefore, necessary to use an integrated rate equation to calculate the initial rate. Eilam and Stein (1974) described an integrated rate equation for the net entry experiment. The initial rate can be estimated by plotting:

$$\frac{t}{c} \quad \text{vs} \quad \frac{-(\ln(1-C/S_o) + (C/S_o))}{C} \qquad (5)$$

where t is the uptake time, C is the internal substrate concentration and S_o is the external substrate concentration. This plot, therefore, shows the rate as a function of the internal concentration as described by the carrier model of Eilam and Stein. By extrapolating the integrated rate plot to zero internal concentration the initial rate of influx measured at different substrate concentrations can then be replotted to obtain the K_m and V_{max} for zero-trans entry. These kinetic parameters reflect the properties of the outside site.

During the inhibition studies in this work a simplification of the zero-trans experiment is used where the rate of entry is calculated according to the equation:

$$V = \frac{f/t}{S} \qquad (6)$$

where f is the fractional filling inside the cell, t is the time and S is the initial substrate concentration. The rates calculated in this way approximate to the initial rate at low values of f. Therefore, uptake times were chosen to give fractional fillings of 40% or less.

In a zero-trans exit protocol, the cells are loaded with radio-labelled substrate until equilibrium is reached. The extracellular substrate is then rapidly diluted with a large quantity of buffer. This method does not give true zero-trans conditions but the small amount of substrate in the trans solution relative to the cis solution leads to minimal error. There is a rapid change in the substrate concentration at the cis face as efflux proceeds so an integrated rate equation treatment is required. The data from zero-trans efflux experiments can be conveniently analysed by an integrated Michaelis-Menten equation (Karlish et al., 1972) which can be rearranged (Baker and Naftalin, 1979) to give:

$$-\ln \frac{S_t/S_o}{S_o - S_t} = \frac{V_{max}}{K_m} \cdot \frac{t}{S_o - S_t} - \frac{1}{K_m} \qquad (7)$$

where S_o is the starting substrate concentration inside the cell and S_t is the internal substrate concentration at time t. This equation is equivalent to a form of the Lineweaver-Burk transformation of the Michaelis-Menten equation:

$$\frac{1}{S} = \frac{V_{max}}{K_m} \cdot \frac{1}{V_o} - \frac{1}{K_m} \qquad (8)$$

Thus by plotting:

$$-\ln \frac{S_t/S_o}{S_o-S_t} \quad \text{vs} \quad \frac{t}{S_o-S_t}$$

a value of $-1/K_m$ can be obtained from the intercept on the ordinate and $1/V_{max}$ is obtained from the intercept on the abscissa.

The kinetic parameters measured by this method reflect the properties of the inside transport site.

1.10.3: Infinite-cis experiments

In the infinite-cis experiments the net flux cis to trans is measured when the substrate concentration in the cis solution is saturating, that is, at least 10 times the zero-trans K_m. The experiment is most easily performed by measuring the time course for net uptake of a single high concentration of substrate. As the uptake proceeds, the internal substrate concentration will increase from zero to equilibrium. Thus the substrate concentration in the trans solution is changing with time and this allows the determination of infinite cis entry parameters. Eilam and Stein (1974) showed that their integrated rate equation for net entry could be applied to the infinite cis protocol (Ginsburg and Stein 1975) so that a plot of:

$$\frac{t}{c} \quad \text{vs} \quad \frac{-(\ln(1-C/S_o + (C/S_o))}{C} \qquad (5)$$

yields a straight line giving $1/V_{max}$ for infinite-cis

entry as the intercept on the ordinate. The intercept on the abscissa will be:

$$-K_m/S_o^2 \ (1 + S_o/\pi)$$

where π is the effective osmotic concentration of the buffer and the K_m can thus be calculated (Holman, 1979). The V_{max} represents the maximum velocity for the outside site whilst the K_m reflects the affinity of the substrate for the inside site.

All the previously-described methods provide a kinetic analysis of a transporter and can determine the symmetry of the binding sites and contribute towards identification of a suitable kinetic model for substrate transport.

1.11: Aims of the project

1. To identify and if necessary, synthesise a radio-labelled non-metabolisable sugar analogue for the *T. brucei* hexose transport system.

2. To carry out a kinetic analysis of the D-glucose transporter, define the type of transport system and the kinetic parameters and establish whether transport is the rate-limiting and possibly the regulatory step in the glycolytic pathway.

3. To investigate the specificity of the

sugar-transporter protein and highlight any differences to mammalian and other transport systems with a view to exploiting suitable differences for the production of anti-trypanosomal agents. These may be either strong selective inhibitors of transport or 'poison' carriers which can release the 'poison' once selectively transported into the trypanosome.

CHAPTER 2: MATERIALS AND METHODS

2.1: Materials

All enzymes and cofactors were supplied by Sigma Chemical Company, Poole, Dorset. Reagents for organic reactions were supplied by Aldrich Chemical Company, Gilingham, Dorset. Ion exchange resins were supplied by BDH Ltd., Poole, Dorset and Whatmann Ltd., Maidstone, Kent. Silica gel-G60 and Silica gel-G60-F preformed thin-layer chromatography plates were from Merck, BDH Ltd., Poole, Dorset. Scintillation fluid was toluene: Triton X-100 (70:30) containing 2,5-diphenyloxazole (5g/l). All other reagents were of analytical grade or the highest grade available.

2.1.1: Radiochemicals

D-(U-^{14}C)-glucose (275mCi/mmol) and 3-O-methyl-D-(U-^{14}C)-glucose (295mCi/mmol) were from Amersham International, UK. (^{3}H)-lithium aluminium hydride (473mCi/mmol) was from New England Nuclear Ltd., Boston, USA.

2.1.2: Buffers

Krebs-Ringer-Phosphate (KRP) buffer was prepared essentially as described by Ryley (1955) thus:

KH_2PO_4	22mM
NaCl	98mM
$MgSO_4$	1mM

KCl	2mM
Final pH	8.00

Krebs-Ringer-phosphate (G) buffer was Krebs-Ringer Phosphate buffer containing 10mM glucose.

2.1.3: Organisms used

Stocks of clones of long slender bloodstream forms of Trypanosoma brucei brucei strain EATRO 427-12/ICI-060 were kindly supplied by Dr Paul Voorheis and stored in sealed capillary tubes in liquid nitrogen. When required, a vial of these clones was thawed out and 10^7 viable trypanosomes used to infect a single rat (See Section 2.2.1). At approximately 72 hours post-infection the rat was anaesthetised with diethyl ether and bled by aortic section into a syringe containing heparin and this blood was mixed with glycerol to a final concentration of 13% (w/v). Small volumes (approximately 25µl) were then drawn into heparinised glass capillary tubes and these tubes, sealed at both ends, were stored in liquid nitrogen. These 'stabilates' (Lumsden and Hardy, 1965) were then used to provide viable trypanosomes for infection into rats (Lumsden, 1972) when cells were required for experimentation.

2.2: Methods

2.2.1: Infection of the host

Prior to infection, one 'stabilate' tube per rat

(2-3 x 10^7 cells) was thawed, the sealed ends removed, and the contents rinsed out using a syringe containing 0.75ml of KRP(G) buffer. The buffer was then injected intraperitoneally into Wistar or Sprague-Dawley rats (250-400g).

2.2.2: Tail count of the host

After approximately 60 hours, the degree of infection of the host was determined by removal of the tip of the tail and dilution of a sample of blood 100-fold in KRP(G) buffer. The number of viable trypanosomes was determined using a Gallenkamp Neubauer haemocytometer-type counting chamber. The time taken to yield 10^9 cells per ml of blood was calculated using a doubling time of 7 hours (Brohn and Clarkson, 1980) and was usually found to be approximately 72 hours.

2.2.3: Isolation of trypanosomes from the host

The method described was adapted from that of Lanham and Godfrey (1970). After approximately 72 hours, the rats were anaesthetised using diethyl ether and bled into 20ml syringes containing 1ml of heparin solution (200 U/ml buffer). Bleeding was carried out at the aorta, situated in the dorsal abdominal cavity. The blood (10-14 ml/rat) was centrifuged for 10 minutes (800 x g, 4^oC) in a bench centrifuge.

The trypanosomes formed a loosely packed layer above the red cells and below the plasma. The plasma

was removed by aspiration and replaced with two thirds the plasma volume of KRP(G) buffer. The trypanosome layer was gently dispersed in the buffer by mild agitation with a pasteur pipette and removed. The wash stage was repeated several times to remove as much of the blood components as possible, The trypanosomes were then purified by passage through a column containing DEAE-cellulose (Whatman DE-52) pre-equilibrated with KRP(G) buffer at 4°C. This column retained the more negatively charged blood cells and platelets whilst allowing elution of a pure trypanosome suspension.

2.2.4: Preparation of a trypanosomal cell free extract

Cell free extracts were prepared as follows:

Freshly prepared trypanosomes were centrifuged for 10 minutes (800 x g, 4°C) in a bench centrifuge and the pellet washed with 400mM triethanolamine buffer, pH 7.4. The centrifugation was repeated and the cells resuspended in the above buffer at a concentration of 10^9 cells/ml of buffer. To 2ml of the cell suspension, 0.5% v/v Triton X-100 was added.

The cells were then sonicated on ice for 90 seconds in 15 second bursts followed by 30 seconds rest, using an Ultrasonics sonicator with a 4mm probe set at 50 watts. The resulting clear suspension contained no intact trypanosomes when examined microscopically and was used in the hexokinase assay.

2.2.5: Preparation of a crude glycosomal extract

A glycosomal extract was also used as a source of trypanosomal hexokinase activity. The method used was a simplification of that described by Panes (1987).

Cells were washed and resuspended in the following disruption buffer: 20mM triethanolamine, 0.5mM phenylmethyl sulphonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 1mM ethylene diamine tetraacetic acid (EDTA), 250mM sucrose, 150mM potassium chloride (minimum volume = 5ml). The suspension was placed in a cooled mortar to which an approximately equal volume of silicon carbide (grade C6-F400) was added to form a thick paste. The paste was then vigorously ground until microscopic examination revealed greater than 95% cell disruption had occurred. The mixture was centrifuged for 5 minutes (100 x g, 4°C). The supernatant was removed and the silicon carbide resuspended in disruption buffer. The centrifugation was repeated twice more and all the supernatants pooled. The supernatants were then centrifuged twice for 20 minutes (1000 x g, 4°C) in a bench centrifuge to remove large cell debris, followed by a further centrifugation for 20 minutes (14,500 x g, 4°C) in a Sorvall RC5-B centrifuge. The resulting glycosome-rich pellet was resuspended in incubation buffer (0.4M triethanolamine, pH 7.4, 0.5% Triton X -100) for use in the hexokinase assay.

2.2.6: Extraction of radio-labelled sugars for thin layer chromatographic (TLC) analysis

Cells containing the transported sugar analogues were 'stopped', centrifuged (See transport assay protocols) and the pellet resuspended in 50 to 100µl of 70% ethanol. The cells were centrifuged for 1 minute (20,000 x g, 0°C) and the supernatant carefully removed from the precipitated pellet. The extract was then applied to silica-gel TLC plates (20µl per track) and dried. The plates were developed in the appropriate solvent system, usually butanol:ethanol:water (52:33:15) or ethyl acetate:ethanol:water (90:10:6). When the solvent front had reached the top, the plate was removed, dried and divided into 4mm sections. Each section was carefully scraped into scintillation vials for counting of radioactivity. When non-labelled sugars were run as standards (5mg/ml) on TLC plates, the location of the bands was visualised by dipping the dried, developed plate into 5% sulphuric acid in methanol and burning off the solvent over a bunsen flame. The presence of sugar was indicated by black charring.

2.3: Transport and metabolism methods

2.3.1: 1-deoxy-D-glycose uptake protocol

Freshly prepared trypanosomes were dispensed into microfuge vials (10^8 cells/vial), each vial sufficient for one duplicate time point.

Immediately prior to the transport experiment the vial was centrifuged and the supernatant removed by aspiration using a microcapillary tube attached to a vacuum line. The cells were resuspended in 1ml of ice cold KRP buffer and recentrifuged. All centrifugations were carried out using an Ole Dich refrigerated micro-centrifuge for 20 seconds (15,000 x g, $0^{o}C$) to ensure rapid processing of the cells. The washed trypanosome pellet was resuspended in 450µl of KRP buffer equilibrated to $37^{o}C$.

The trypanosomes (450µl) were placed in a small glass vessel enclosed in a water jacket maintained at $37^{o}C$. This provided good thermal contact and allowed rapid equilibration after addition of any substances not maintained at $37^{o}C$. The cells were pre-incubated for 30 seconds and incubation commenced by addition of 50µl of the appropriate concentration of radio-labelled 1-deoxy-D-glucose containing 0.5µCi of radio-label.

After the appropriate time interval duplicate 200µl samples were removed using a multichannel pipette and dispensed into vials containing 1ml of ice cold KRP buffer to stop the uptake.

The vials were centrifuged, the supernatants removed by aspiration and the pellets resuspended on a vortex mixer in 1ml of ice-cold KRP buffer. After a further centrifugation, the supernatants were removed taking great care to remove all small drops adhering to the sides of the vial. At the end of the experiment all

pellets were resuspended in 0.5ml of distilled water and allowed to stand at room temperature for one hour to lyse the cells and release the radio-label. After lysis had occurred, 0.45ml samples were added to vials containing 5ml of scintillant for counting of radioactivity in a Packard Tricarb liquid scintillation counter.

Carry-over of non-transported radio-label was estimated by addition of radio-label to cells equilibrated at 2°C followed by immediate removal of samples to ice-cold stopping solution. Zero values were then subtracted from total CPM values measured.

Later kinetic and inhibitor studies on 1-deoxy-D-glucose used the improved protocol for zero-trans uptake of 6-deoxy-D-glucose (Section 2.3.3).

2.3.2: 1-deoxy-D-glucose efflux protocol

For study of 1-deoxy-D-glucose efflux, cells were prepared as described for uptake, up to the incubation with the radio-label. The cells were incubated for three minutes with the radio-label in a microfuge tube, then centrifuged and the pellet resuspended in 1ml of ice-cold KRP buffer. The cells were rapidly centrifuged and resuspended in 0.5ml of KRP buffer at 37°C and placed in the glass incubation vessel. This process took 30 seconds to complete. At the appropriate time after resuspension, efflux was stopped by removal of 200µl samples to 1ml of ice-cold stopper and processed as described for the uptake experiment.

Carry-over of extracellular radio-label was estimated by continuing efflux for 10 minutes before stopping the reaction. This zero-value was subtracted from all other values.

2.3.3: Zero-trans entry protocol

Freshly prepared trypanosomes in KRP(G) buffer were dispensed into the appropriate number of 3.5ml polypropylene tubes containing 1ml of cold KRP buffer (5×10^7 cells/tube, 1 tube = 1 uptake determination). An equivalent number of tubes were prepared containing 2ml of ice-cold KRP buffer + 0.1% phloridzin for use as stopping solution. All the above tubes were maintained close to 0^oC in an ice/water bath.

Solutions of radio-labelled 6-deoxy-D-glucose were prepared by the addition of 10µl of a 10-fold dilution of stock radio-label (specific activity = 8.53Ci/m mol) into the appropriate number of microfuge tubes (1.2µCi/ tube). For time-course experiments, a single concentration of unlabelled 6-deoxy-D-glucose was prepared (10 times the desired final concentration). A range of concentrations was used when carrying out kinetic constant estimations. To each microfuge tube, 10µl of the appropriate concentration was added. All tubes containing labelled 6-deoxy-D-glucose were equilibrated to 20^oC. These preparations, prior to the commencement of the assay, removed the need for precise pipetting during the course of the assay.

The assay was commenced by centrifugation of one tube containing trypanosomes (12,000 x g, 0°C, 20 seconds) followed by resuspension in 1ml of cold KRP buffer. The centrifugation was repeated and the washed cells resuspended in 80µl of KRP buffer pre-equilibrated to 20°C. To begin the incubation the cells were pipetted into the appropriate tube containing the labelled sugar and incubated for the required period of time (final volume = 100µl). Uptake was stopped by rapid addition of the cells to 2ml of ice-cold stopper solution. For incubation times of less than 10 seconds, a metronome set at two beats per second was used for timing purposes. The 'stopped' cells were centrifuged, the pellet resuspended in 1ml of stopping solution and the centrifugation repeated. The supernatant was then carefully removed by aspiration ensuring the removal of all adhering droplets. After all uptake estimations had been completed, the cell pellets were lysed by resuspension in 0.5ml of distilled water and left to stand at room temperature for 30 minutes The cell lysate was then dispensed into scintillation vials (0.45ml lysate: 5ml scintillant) for counting of radioactivity.

Carry-over of extracellular radio-label was estimated by direct addition of cells resuspended in 80µl of cold KRP buffer to stopper containing the radio-labelled 6-deoxy-D-glucose. The 'zero-time' counts were subtracted from all subsequent uptake values.

Equilibrium values were obtained by incubation of cells for 60 to 120 seconds with labelled 6-deoxy-D-glucose. Using the equilibrium value, the fractional filling of the cells was calculated as follows:

$$f = \frac{CPM_t - CPM_o}{CPM_\infty - CPM_o}$$

Uptake was expressed in units of concentration sec^{-1}(C). C was calculated as follows:

$$C = \frac{f \cdot S_o}{t}$$

where S_o is the initial concentration outside the cells and t is the incubation time in seconds.

For single time point assays, an incubation time of three seconds was used to estimate the initial rate of uptake. This represented a fractional filling of approximately 40%.

2.3.4: Zero-trans exit protocol

Prior to commencement of the assay, the appropriate number of tubes containing 5×10^7 trypanosomes and tubes containing 2.5ml of stopping solution were set up and maintained close to 0^oC. Appropriate concentrations of labelled 6-deoxy-D-glucose were prepared by the addition of 3µl of 8.33 times the desired final concentration of unlabelled 6-deoxy-D-glucose to a microfuge tube containing 2µl of undiluted radio-label

(2.4μCi/tube). The final concentrations used were 1, 2, 5 and 10mM. These tubes were equilibrated to 20°C.

The assay was commenced by washing the cells to remove extracellular D-glucose as described in the zero-trans entry protocol. The washed cells were resuspended in 20μl of KRP buffer maintained at 20°C. The cells were then added to the appropriate radio-label (total volume = 25μl) and incubated for 5 minutes to ensure full equilibration. Efflux was commenced by rapid addition of the cells to 1.225ml of vigorously stirring KRP buffer contained in the same glass vessel used in the 1-deoxy-D-glucose uptake protocol (Section 2.3.1). The mixture was incubated for the required time, then 125μl was dispensed into 2.5ml of ice-cold stopping solution. Timing was aided by the use of a metronome set at two beats per second. The time intervals used ranged from 3 to 18 seconds thus building up a time-course of efflux at each concentration. The stopped cells were washed and processed for counting as described in the zero-trans entry protocol.

A zero-time point for efflux was obtained by rapid addition of the preincubated cells to 1.255ml of ice-cold stopping solution followed by immediate removal of 125μl to 2.5ml of stopping solution. This reproduced the dilutions occuring for all the other time points. Zero-time points were determined for each concentration used but were found to be approximately equal indicating that full equilibration of the radio-label had taken place.

A time point of 60-90 seconds after efflux had commenced was determined to account for trapped extracellular radio-label.

To obtain the kinetic parameters under these conditions, all the time points at each concentration were fitted and plotted using the integrated rate equation of Karlish et al., (1972) (see Results 4.3.4).

2.3.5: Equilibrium exchange entry protocol

The equilibrium exchange protocol was essentially the same as the zero-trans entry protocol except that cells were pre-equilibrated with the appropriate concentration of unlabelled substrate for three minutes prior to the addition of the same concentration of labelled substrate.

Cells were washed as described previously (Section 2.3.3) and resuspended in 75µl of KRP buffer at 20°C (5×10^7 cells). To commence preincubation 10µl of 8.5 times the desired concentration of 6-deoxy-D-glucose was added and the cells mixed using a vortex mixer. To follow the uptake of radio-label, 15µl of the final desired concentraton of labelled 6-deoxy-D-glucose was rapidly added. The 15µl aliquot was made up of 5µl of three times the desired concentration of 6-deoxy-D-glucose plus 10µl of a 10-fold dilution of radio-labelled 6-deoxy-D-glucose (1.2µCi/tube).

The cells were incubated for the desired time (3 or 4 seconds) and stopped by rapid addition of the cells

to 2ml of ice-cold stopping solution. The cells were then processed as previously described in Secion 2.3.3. An equilibrium value for uptake of radio-label was obtained by incubating the cells for 90 seconds prior to the addition to stopper.

A 'time-zero' value was obtained by addition of the pre-equilibrated cells directly to ice-cold stopper containing 15µl of radio-labelled 6-deoxy-D-glucose. This value for trapped extracellular radioactivity was subtracted from all other values.

As there was no net flux of 6-deoxy-D-glucose into the cells under this protocol, the accumulation of radio-label closely approximated to a simple exponential curve. The time point used was thus not critical and the initial rate of uptake was calculated using the following relationship:

$$V_o = \frac{-\ln(1-f).S_o}{t}$$

where S_o is the substrate concentration, f is the fractional filling and t is the incubation time.

To determine the kinetic parameters under equilibrium exchange conditions, a concentration range of 0.1 to 10mM 6-deoxy-D-glucose was used.

2.3.6: Infinite-cis entry protocol

The infinite-cis entry protocol was effectively a

time-course for uptake at a saturating external concentration of 6-deoxy-D-glucose. During this one time-course, the external substrate concentration remained saturating and the internal substrate concentration was continuously increasing with time. Correct analysis of the uptake profile can yield the kinetic parameters under infinite-cis entry conditions as described below.

The substrate concentration used was 40mM 6-deoxy-D-glucose, this being much higher than the zero-trans entry or exit K_m values. The uptake protocol was exactly as described for the zero-trans entry protocol (Section 2.3.3).

The time course was replotted using the following form of the integrated rate equation:

$$t/C \quad vs \quad \frac{-(\ln(1-C/S_o) - C/S_o)}{C}$$

The V_{max} and K_m were obtained as described in the results section.

2.3.7: Substrate respiration using the oxygen electrode

The trypanosomal substrate respiration rate was determined by measuring the rate of oxygen consumption using a 'Clark' oxygen electrode (Beechey and Ribbons, 1972) set up using a 12μm teflon membrane. Calibration of the electrode was carried out using KRP buffer through which air was bubbled for 1 hour.

Zero percent oxygen was determined by the addition of a few grains of sodium dithionite ($Na_2 S_2O_4$). The position of the chart recorder pen at 0% oxygen was equivalent to that obtained when short-circuiting the chart recorder. One hundred percent oxygen was assumed to be equivalent to a concentration of 240µM oxygen (Robinson and Cooper. 1970).

Water of an appropriate temperature (37°C or 20°C) was circulated through the jacket enclosing the electrode. Stock KRP buffer was also maintained at the appropriate temperature. The final volume of the solution contained in the electrode was 3ml and the mixture was stirred at a constant speed.

Trypanosomes were aliquoted into microfuge tubes (3×10^8 cells per tube) and when required were washed by centrifugation (12,000 x g, 20 seconds, 2°C) and resuspension in KRP buffer to remove extracellular D-glucose. The cells were resuspended in 300µl of buffer and placed into the electrode containing 2.6ml of buffer (10^8 cells/ml). The appropriate concentration of substrate was then added to the chamber via the small hole in the lid using a 100µl Hamilton syringe.

The rate of substrate consumption was determined from the gradient on the chart relating to oxygen consumption (1 molecule oxygen = 1 molecule substrate).

For inhibition experiments, the same procedure was carried out except 100µl of the appropriate inhibitor concentration was added immediately prior to substrate addition. The volume of buffer was adjusted to maintian

the final total volume to 3ml.

2.3.8: Hexokinase assay protocol

Hexokinase activity in T. brucei was determined by measuring the production of adenosine diphosphate (ADP) in a coupled system containing pyruvate kinase and lactate dehydrogenase at 25°C (Figure 11). The production of NAD^+ was measured using a Cecil CE-212 ultraviolet spectrophotometer at a wavelength of 340nm. The assay buffer (pH 7.4) contained 0.4M triethanolamine, 2mM EDTA, 4mM $MgCl_2$ 4mM KCl, 0.5mM ATP, 0.5mM phosphoenolpyruvate, 0.25mM NADH, 5U. of pyruvate kinase and 5U. of lactate dehydrogenase.

Trypanosomal hexokinase was assayed using either a crude glycosomal preparation or a sonicated cell lysate (Sections 2.2.5 and 2.2.6). The amount of hexokinase preparation added per assay was equivalent to that produced by 10^8 cells. A correction for NADH oxidase activity was determined by ommission of phosphoenolpyruvate and the two coupling enzymes. The rate was found to be less than 0.5% of maximal hexokinase activity in the presence of D-glucose.

2.3.9: Intracellular D-glucose assay protocol

The method used was the glucose oxidase/peroxidase O-dianisidine procedure.

Trypanosomes were incubated at room temperature for 15 minutes in KRP(G) buffer. The cells were then washed

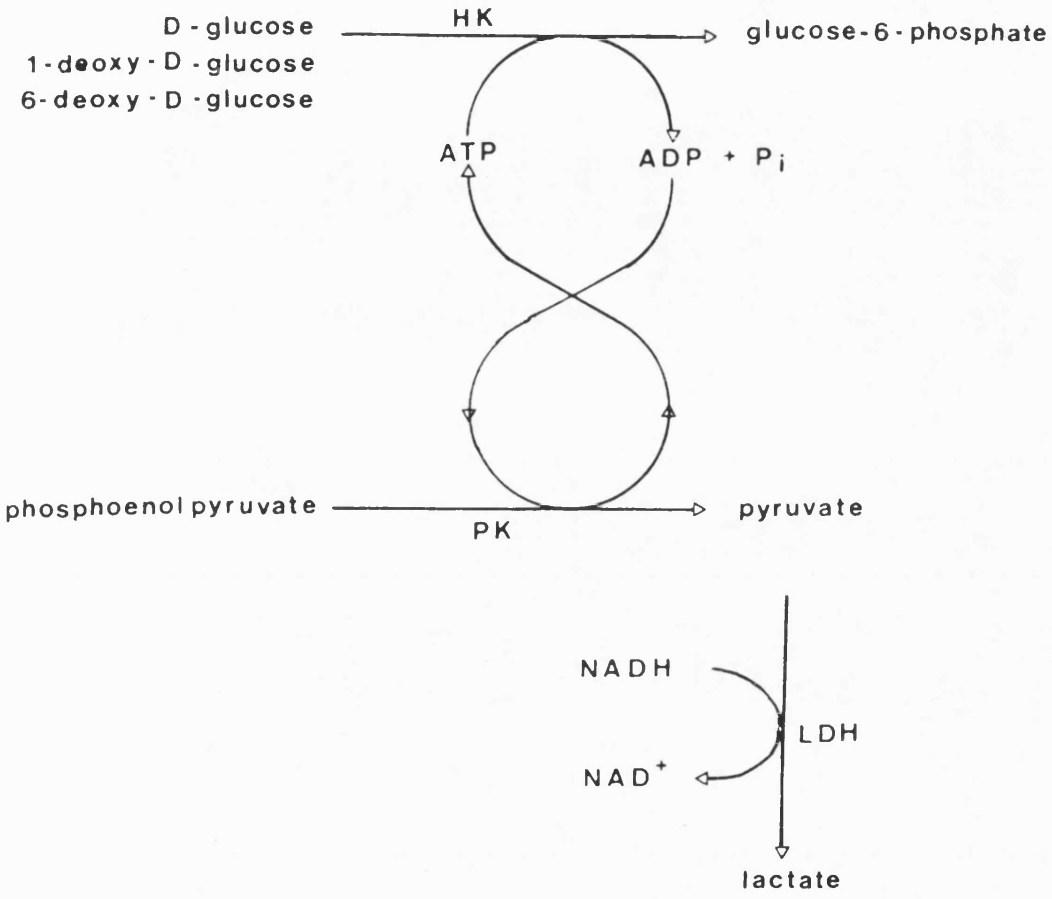


Figure 11: Reaction scheme for the assay of ADP production coupled to the formation of NAD^+. HK = hexokinase as present in T. brucei cell preparation, PK = pyruvate kinase, LDH = Lacetate dehydrogenase.

twice by centrifugation and resuspension in ice-cold buffer. Cells were finally resuspended in 2ml of buffer at a concentration of 5 x 10^9 cells/ml. Triton X-100 was added (0.1%) and the cells were sonicated on ice for 90-120 seconds in 15 second bursts followed by 30 seconds rest using an Ultrasonics sonicator fitted with a 4mm probe set at 50 watts. The resulting lysed cell suspension was used to determine the intracellular D-glucose concentration.

The assay procedure utilized the following reactions:

1. D-glucose + H_2O + O_2 $\xrightarrow{\text{glucose oxidase}}$ Gluconic acid + $2H_2O_2$

2. H_2O_2 + 0-dianisidine reduced form (colourless) $\xrightarrow{\text{peroxidase}}$ 0-dianisidine oxidised form (brown) + H_2O

The assay constituents were in the form of a pre-weighed capsule containing 500 international units of glucose oxidase (Aspergillus niger), 100 purpurogallin units of horseradish peroxidase and buffer salts. The capsule was dissolved in 100ml of distilled water to which 1.6ml of 0-dianisidine solution (2.5mg/ml) was added. The assay mixture consisted of 900µl of enzyme/colour/buffer mixture and 100µl of lysed trypanosomal extract. The cuvettes were mixed and left in the dark for 30 minutes to allow the colour to develop. For reference a standard D-glucose concentration curve was made up (0-2mM).

The colour change was read using a Cecil CE-212 spectrophotometer set at a wavelength of 450nm.

CHAPTER 3: CHEMICAL SYNTHESIS OF SUGAR ANALOGUES

3.1: Synthesis of labelled 6-deoxy-D-glucose using tritiated lithium aluminium hydride

3.1.1: Methyl-6-Chloro-6-deoxy-D-glucopyranoside synthesis

The first stage of the synthesis of 6-deoxy-D glucose followed a modification of the method used by Evans and Parrish (1972) for the synthesis of 6-Chloro-6-deoxy-α-D-glucoside:

Methane sulphonyl chloride (37.5ml, 56.5g, 5 moles) was added to a stirred solution of 19.4g of methyl-α-D-glucopyranoside in 200ml of N, N-dimethylformamide cooled to 18°C in a water bath. The temperature was slowly increased to 70°C and maintained for 16 to 18 hours. N-propanol (37.5ml) was then added to the hot solution which was maintained at 70°C for a further 3 hours. The product was concentrated under reduced pressure at a temperature not exceeding 55°C using first a water aspirator, then an oil pump. The cooled solution was diluted with 500ml of water and left for 1 hour at room temperature to hydrolyse formate esters formed in the reaction. The product was again concentrated under reduced pressure, the syrup dissolved in 10ml of ethyl acetate and then applied to a 6 x 50cm column of silica-gel. The product was eluted using ethyl acetate: ethanol:water, (90:10:6), the appropriate fractions being pooled and concentrated under reduced pressure. This

yielded 12g (58.8%) of methyl-6-chloro-6-deoxy-D-glucopyranoside which was further purified by recrystallization from ethyl acetate: Final yield 10.9g (53.4%), melting point: 111°C (Literature melting point 112°C-113°C), R_f = 0.50 on silica-gel pre-coated glass plates developed in ethyl acetate:ethanol:water (90:10:6).

3.1.2: Protection of the free hydroxyl groups using dihydropyran

The method used by Evans and Parrish (1972) involved the simple reduction of the chloro-sugar using lithium aluminium hydride ($LiAlH_4$). This was not practical when using labelled $LiAlH_4$ as tritium exchange with the free hydroxyl-groups on the chloro-compound would have wasted most of the label.

The first method of protection of the hydroxyl-groups used the compound dihydropyran, previously used for similar protection reactions (Wolfrom et al., 1968). The most suitable molar ratio of reactants was 5g of 6-chloro-6-deoxy-α-methyl-glucoside dissolved in 125ml of dry N, N-dimethylformamide to which 40ml of dihydropyran was added along with catalytic amounts of toluene sulphonyl chloride. (Molar ratio of dihydropyran to each hydroxyl group 8:1).

After one hour at room temperature approximately equal amounts of di- and tri-substituted sugar were present as judged by TLC and after a further 10 hours, a single band corresponding to the expected position of

2,3,4-tetrahydropyranyl-6-chloro-6-deoxy-methyl-α-D-glucopyranoside was obtained (R_f = 0.85 developed in ethyl acetate:ethanol:water, 90:10:6). Dichloromethane (100ml) was added to the reaction mixture and shaken with an equal volume of 1M potassium hydrogen carbonate. The aqueous layer was removed and the organic phase washed several more times, then concentrated under reduced pressure. No crystallization of the resulting syrup occured. This was thought to be due to the product being a mixture of stereoisomers. Subsequent TLC analysis supported this assumption by revealing several bands when developed in petroleum ether:ethyl acetate (4:1).

Approximately 8g (72.9%) of syrup was produced, half of which was purified on a silica-gel column eluted with the solvent mixture: petroleum ether:ethyl acetate (1:5). Of the three major isomers visualised, fractions containing only one product for two of the three bands were pooled and evaporated to dryness. These were then left to crystallize in the fridge for use in subsequent labelling of the protected sugar. The remaining syrup was used in a trial reduction using unlabelled $LiAlH_4$.

3.1.3: Protection of the free hydroxyl-groups using tertiary-butyl-dimethyl-silyl-chloride

Tertiary-butyl-dimethyl-silyl-chloride (t-BDSCl) was investigated as a potential protecting agent due to its symmetrical structure. Such a structure avoids the

problem of producing stereoisomers and would allow the synthesis of 'clean' crystalline products. The conditions used in the reaction were as follows:

To a solution of 100mg of 6-chloro-6-deoxy-α-methyl-D-glucoside in 2ml of N,N-dimethyl formamide, was added 425mg of t-BDSCl. (2 equivalents/hydroxyl group) followed by 380mg of imidazole (imidazole:t-BDSCl; 2:1). All additions were carried out using thoroughly dried glassware under a stream of dry nitrogen gas.

The mixture was left at room temperature for 12 hours. The progress of the reaction was monitored using TLC analysis developing the plates in ethyl acetate: petroleum ether (1:9). This revealed equal proportions of two new products: R_f 0.45 and 0.49. Due to the close proximity of the bands to each other, a chromatotron was used for the isolation of the products. This automated TLC procedure produced a successful separation of the two compounds which were then evaporated to dryness, crystallized and analysed using NMR spectrometry.

The ratio of the silyl-compound to the sugar revealed that both isolated compounds were sugar residues with two silyl-groups attached, each one presumably being a different isomer. This suggested that there may have been steric problems with the protection of all three hydroxyl-groups with the silyl compound. The reaction was allowed to proceed for seven days gradually increasing the temperature to 35°C. During this time, a small amount of product (R_f 0.75, ethyl acetate:petroleum ether,

1:9) was produced. This was assumed to be the fully-protected sugar compound. However, due to the low yield and the length of time taken for the reaction to proceed, the method of protection of the free hydroxyl-groups using t-BDSCl was not further investigated.

3.1.4: Reduction of 2,3,4-tetrahydropyranyl-6-chloro-6-deoxy-methyl-α-D-glucoside

The protected glucoside (3.95g) was dissolved in 10ml of freshly distilled tetrahydrofuran (THF). This solution was then carefully added to a vigorously-stirred suspension of 0.6g $LiAlH_4$ dispersed in 40ml of THF. The mixture was refluxed for 18 hours and, after cooling, a further 0.4g of $LiAlH_4$ in 10ml of THF was added. After an additional 18 hours of refluxing any remaining $LiAlH_4$ was neutralised by the very careful dropwise addition of 20ml of 15% sodium hydroxide solution and removal of the insolulable salts by filtration. The aqueous solution was washed four times using 50ml of dichloromethane to extract the protected sugar into the organic phase. Anhydrous sodium sulphate was added to dry the solvent. The solution was finally evaporated to dryness to yield 3.2g (86%) of crude product. The yield assumes that no impurities were present.

3.1.5: Mild acid-hydrolysis of 2,3,4-tetrahydropyranyl-6-deoxy-α-methyl-D-glucoside

A solution of 10ml of 50% ethanol containing 1g of

the reduced sugar compound was made up, to which 6g of pre-washed Dowex-50 (H^+ form) ion-exchange resin was added. The mixture was heated for 4 hours at 70°C then allowed to cool. The ion-exchange resin was removed by filtration and thoroughly washed with distilled water. The resulting aqueous solution was evaporated to dryness under reduced pressure. The syrup was then dissolved in solvent (ethyl acetate:ethanol:water, 90:10:6) and purified using a 10cm x 2.5cm silica-gel column eluting with the above solvent mixture. Thin layer chromatography revealed three products: R_f = 0.42, 0.31 and 0.15 (solvent; ethyl acetate:ethanol:water).

The product: R_f = 0.15 corresponded to standard 6-deoxy-D-glucose. The two remaining products were crystallized and analysed using nuclear magnetic resonance (NMR) spectrometry.

The major product (R_f = 0.31, 42.7%) was 6-deoxy-D-α-methyl-glucoside and the minor product was unreacted 6-chloro-6-deoxy-α-methyl-D-glucoside.

3.1.6: Strong acid-hydrolysis of 6-deoxy-α-methyl-D-glucoside

A solution of 160mg of 6-deoxy-α-methyl-D-glucoside dissolved in 10ml of 1M hydrochloric acid was refluxed for 4 hours. The solution was cooled and neutralised with pre-washed Amberlite IRA-93 (OH^- form) ion-exchange resin. The neutralised solution was evaporated to dryness under reduced pressure and analysed using TLC.

The analysis revealed a single product (R_f = 0.14) corresponding to standard 6-deoxy-D-glucose (solvent mixture; ethyl acetate:ethanol:water, 90:10:6).

Further purification by paper chromatography and crystallization of the product was not attempted.

3.1.7: Synthesis of radio-labelled 6-deoxy-D-glucose

An attempt was made to radio-label the previously isolated isomer of 2,3,4-tetrahydropyranyl-6-chloro-6-deoxy-α-methyl-D-glucoside by reduction using tritiated $LiAlH_4$. Unlabelled $LiAlH_4$ (1.6mg) was rapidly added to 2ml of freshly distilled THF. The suspension was then added to the ampoule containing the tritiated $LiAlH_4$ (0.4mg, 5mCi, specific activity = 473.8 mCi/mmole) immediately after opening. The suspension was immediately transferred to a reaction vessel containing 10mg of the sugar residue and refluxed for 48 hours. All the previously described operations were carried out under a stream of dry nitrogen gas using thoroughly dried glassware.

After reflux for the required time, the mixture was cooled and a small sample neutralised by the addition of a few drops of distilled water. Subsequent TLC analysis revealed that no labelled product was detectable. The reaction mixture was refluxed for a further 48 hours, cooled and unreacted $LiAlH_4$ decomposed by the addition of distilled water. Chromatography again revealed that no radio-labelled product was present. (See discussion).

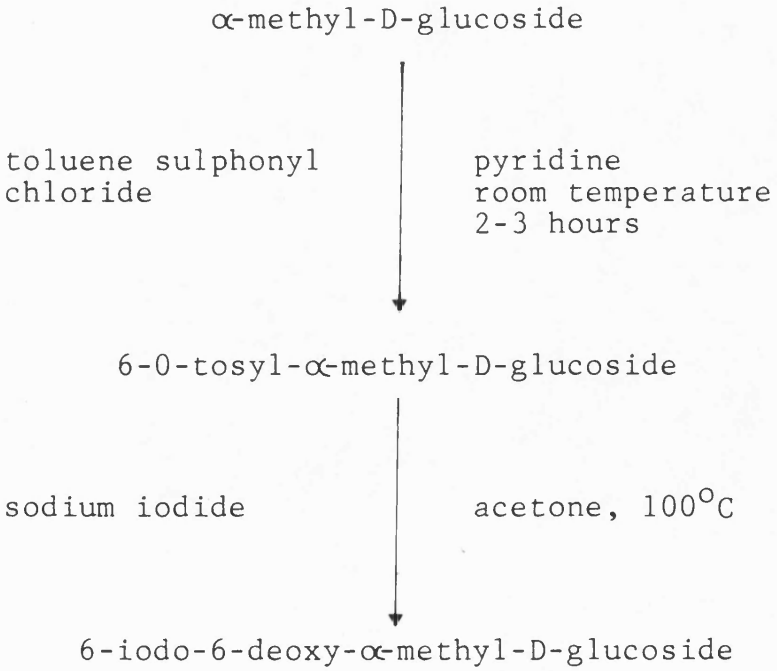
A repeat of the reduction using tritiated-$LiAlH_4$ was not able to be carried out due to the supply of $LiAlH_4$ being permanently discontinued by the sole supplier.

3.2 Synthesis of 6-deoxy-D-glucose using the catalysed halogen/tritium exchange method

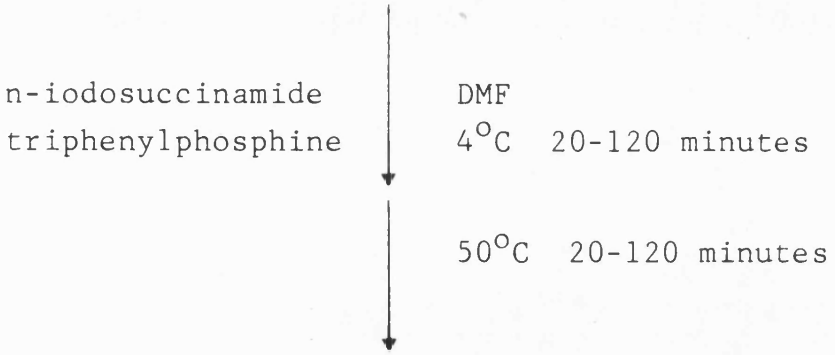
The alternative method of synthesis of radio-labelled 6-deoxy-D-glucose investigated was the catalysed halogen/tritium exchange service provided by Amersham International, Amersham, UK. The recommended substrate for the exchange procedure was 6-Iodo-D-deoxy-α-methyl-D-glucoside.

Several procedures for the synthesis of the required iodo-compound were attempted as indicated by the reaction schemes shown below:

A)

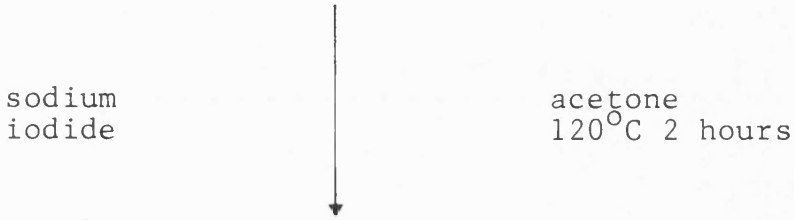


B) α-methyl-D-glucoside



6-iodo-6-deoxy-α-methyl-D-glucoside

C) 6-Chloro-6-deoxy-α-methyl-D-glucoside



6-iodo-6-deoxy-α-methyl-D-glucoside + NaCl

Several problems were encountered with methods A) and B) such that no product could be readily identified or purified (See Discussion). Method C) was however successful and is described in detail below.

3.2.1: Halide exchange of 6-chloro-6-deoxy-α-methyl-D-glucoside

This method of producing the iodo-sugar utilised 6-chloro-6-deoxy-α-methyl-D-glucoside prepared previously (Section 3.1.1). An appropriate amount of 6-chloro-6-deoxy-α-methyl-D-glucoside was thoroughly dried overnight

in a dessicator and 500mg added to 25ml of dry acetone. To this, 8g of sodium iodide was added and vigorously shaken until the acetone was saturated with the salt. The solution was then placed in an airtight glass tube and heated in an oven for 2 hours at 120°C. The solution was then cooled to room temperature. At this stage, a small amount of solid material was visible in the glass tube. This was assumed to be insoluble sodium chloride formed during the reaction.

The acetone was evaporated under reduced pressure leaving a blue/brown precipitate which was dissolved in 10ml of distilled water. To this, approximately 15g of pre-washed Amberlite MB-1 ion-exchange resin was added. The resin was then removed by filtration and thoroughly washed with distilled water. The aqueous solution was concentrated under reduced pressure and the resulting syrup dissolved in a small volume of ethyl acetate: ethanol:water, 90:10:6). This was then applied to a 2.5 x 20cm column of silica-gel and eluted with the above solvent mixture under mild pressure.

The fractions containing the sugar compound were pooled and concentrated under reduced pressure to give a solid material which was further purified by recrystallization from ethyl acetate. Analysis by mass-spectrometry revealed the crystalline product as 6-iodo-6-deoxy-α-methyl-D-glucoside, yield = 25mg (3.5%), R_f = 0.41 (ethyl acetate:ethanol:water. 90:10:6) (Chloro-sugar R_f = 0.37).

3.2.2: Catalytic halogen/tritium exchange

The product (22.5mg) was sent to Amersham for the catalytic halogen/tritium exchange procedure to be carried out. The reaction yielded 630mCi of 6-deoxy-α-methyl-D-glucoside, specific activity = 8.53 Ci/mmol.

3.2.3: Acid hydrolysis of 6-deoxy-α-methyl-D-glucoside

A 10mCi aliquot of the product was removed and evaporated to dryness under a stream of nitrogen gas. The labelled compound was dissolved in 2ml of 1M hydrochloric acid. The solution was then refluxed for 4 hours, cooled and neutralised using Amberlite IRA-93 (OH^-) ion-exchange resin after dilution with 10ml of distilled water. The aqueous solution was then concentrated under reduced pressure. TLC analysis revealed a single radioactive product, R_f = 0.17 (Figure 12). The plates were developed in ethyl acetate:ethanol:water, (90:10:6).

The R_f value for the radio-labelled product was the same as standard 6-deoxy-D-glucose solution.

3.2.4: Purification by preparative paper chromatography

The radio-labelled 6-deoxy-D-glucose was purified using paper chromatography to remove any potential non-radioactive contaminants. A 30cm piece of Whatmann-3MM filter paper was cut and folded, to which a 1cm wide box was pencilled near the top of the paper to within 3cm of

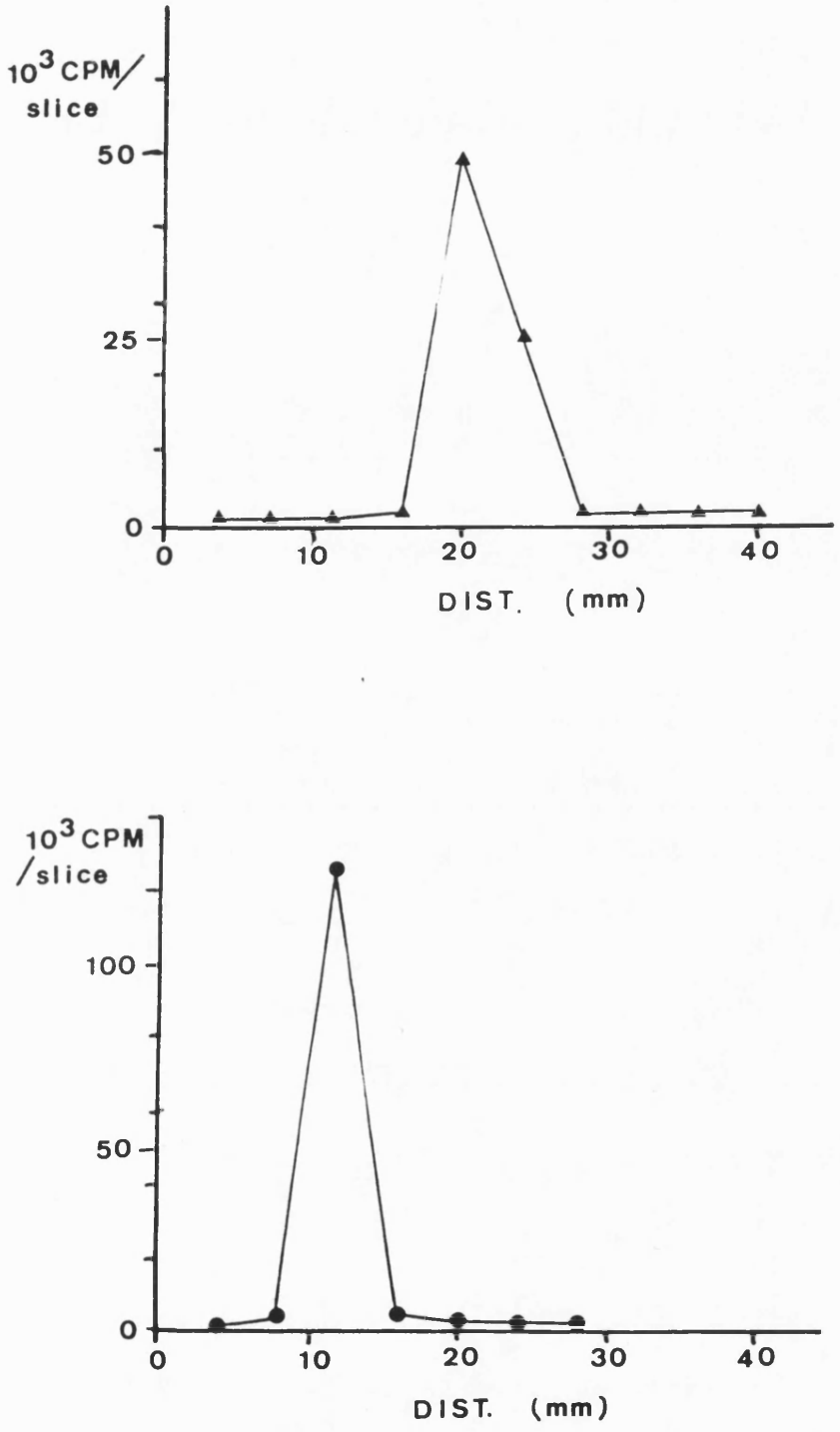


Figure 12: TLC analysis of labelled 6-deoxy-α-methyl-glucoside (▲) and labelled 6-deoxy-D-glucose (●). Counts per minute are shown as per slice from the TLC plate vs. the distance of the slice from the origin. Solvent system: ethyl acetate:ethanol:water, (90:10:6).

either edge. The aqueous solution containing the sugar (200µl) was carefully applied within the box and allowed to dry. The flask containing the radio-labelled sugar was rinsed with 200µl of distilled water which was then applied to the box and up to each edge of the paper. The paper was developed overnight in butanol:ethanol:water, (52:33:15) and dried in a fume cupboard. The strips at each edge of the paper were removed and divided into 1cm sections. These were then placed in scintillation vials and the radioactivity counted to reveal the location of the radio-label on the paper. The appropriate area of paper was cut out and eluted with distilled water. The eluant was aliquoted into microfuge vials and stored at -20°C. Final yeild, 6.37mCi (63.7%) of 6-deoxy-D-glucose in 3.73ml of distilled water (210µM).

3.3: Synthesis of radio-labelled 1-deoxy-D-glucose

The synthesis of 1-deoxy-D-glucose was adapted from the method of Ness et al., (1950):

1mCi of D-(U-^{14}C)-glucose in ethanol:water. 9:1 was evaporated to dryness and stored overnight in a dessicator. The following day, 1ml of acetic anhydride and 1ml of pyridine were added to the D-glucose and the flask shaken for 24 hours at room temperature. The product was extracted into 50ml of chloroform and washed first with 50ml of 100mM hydrochloric acid then 100ml of distilled water. The aqueous phases were washed with 20ml of chloroform which was pooled with the chloroform

extract. The reaction yielded 99% labelled D-glucose pentacetate which was dried over sodium sulphate overnight. The chloroform extract was evaporated to dryness under reduced pressure and 1ml of 45% hydrogen bromide in glacial acetic acid was added. The mixture was shaken for 2 hours at room temperature then extracted into 50ml of chloroform. The chloroform extract was washed twice with 100ml of distilled water followed by 100ml of ice-cold saturated sodium hydrogen carbonate solution. The yield was 64% 1-bromo-2,3,4,6-tetra-acetyl-D-glucose. The extract was dried overnight and evaporated under reduced pressure. The 1-bromo-tetra-acetyl-D-glucose was dissolved in 5ml of diethyl ether and added dropwise to 100mg of $LiAlH_4$ suspended in 2ml of diethyl ether. The suspension was stirred for 30 minutes at room temperature in the dark followed by very careful dropwise addition of distilled water to decompose excess $LiAlH_4$. The water phase was then separated from the diethyl ether phase and the diethyl ether washed with 50ml of distilled water. Pre-washed Amberlite MB-1 ion-exchange resin was added to the aqueous solution to remove the salts that were produced. The ion-exchange resin was separated from the solution by filtration and washed with distilled water. The aqueous phase was evaporated to dryness under reduced pressure and resuspended in 150µl of distilled water. TLC analysis revealed a major product R_f = 0.51 developed in butanol: ethanol:water, (52:33:15) corresponding to standard

1-deoxy-D-glucose solution.

The radio-labelled product was purified using paper chromatography as previously described (Section 3.2.4). The elution profile is shown in Figure 13.

The final purification yielded 503μCi of 1-deoxy-D-glucose, specific activity 275mCi/mmol. Overall percentage yield = 50.3%.

3.4: Synthesis of 6-chloro-6-deoxy-D-glucose

The product 6-chloro-6-deoxy-α-methyl-D-glucose previously synthesised (Section 3.1.1) was hydrolysed to produce 6-chloro-6-deoxy-D-glucose by the following procedure: 1 gram of 6-chloro-6-deoxy-α-methyl-D-glucoside dissolved in 10ml of 1M hydrochloric acid was refluxed for 6-8 hours. The solution was cooled and neutralised with Amberlite IRA-93 (OH^-) ion-exchange resin. The resin was separated by filtration and washed with distilled water. The aqueous solution was concentrated under reduced pressure and purified on a 2.5 x 20cm silica-gel column. The 6-chloro-6-deoxy D-glucose was eluted with butanol:ethanol:water (52:33:15), the relevant fractions pooled and concentrated to dryness. Further purification by recrystallization from ethyl acetate yielded 0.27g of white feathery crystals of 6-chloro-6-deoxy-D-glucose. Yield = 27%.

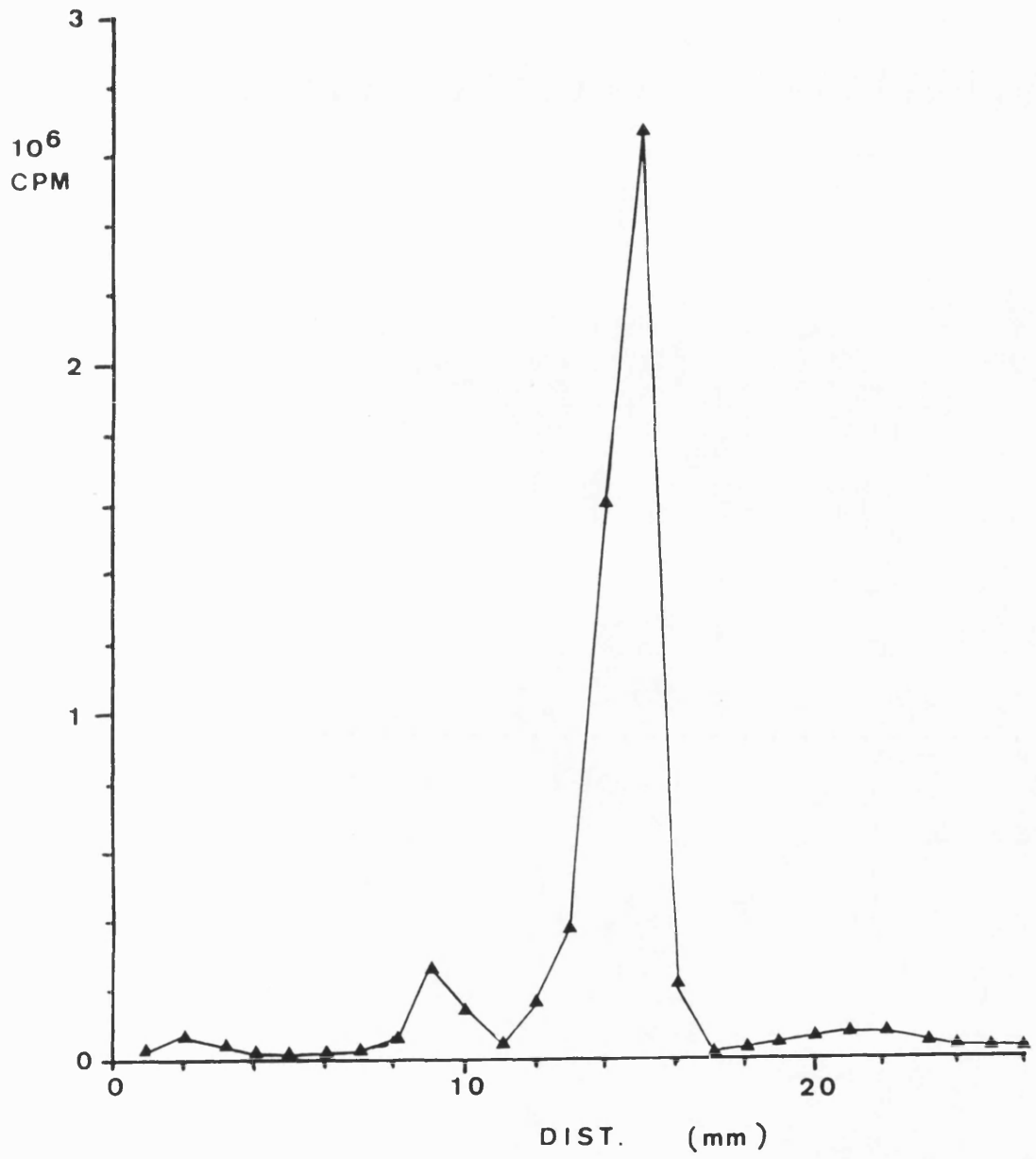


Figure 13: Elution profile of the paper chromatogram of the final product in the synthesis of 1-deoxy-D-glucose. The paper corresponding to the large peak was removed and the product eluted with water. This product was 1-deoxy-D-glucose. Solvent system: Butanol:ethanol: water, (52:33:15).

CHAPTER 4: RESULTS

4.1: Substrates of T. brucei

4.1.1: Specificity of substrate metabolism

The oxidation of substrates by the bloodstream form of Trypanosoma brucei was investigated using a Clark type oxygen electrode to measure the rate of oxygen consumption as a function of substrate metabolism.

The results shown in Table 2 confirmed that the substrates metabolised by T. brucei were D-glucose, D-mannose, D-fructose and glycerol. The rate of oxygen consumption by trypanosomes incubated with a 1mM concentration of each of these substrates was approximately the same. The background rate of oxygen consumption without substrates was similar to the rates obtained using D-galactose, α-glycerol phosphate and inosine, these potential substrates therefore not being metabolised to any extent.

The results in Table 2 were confirmed by microscopic examination. Cell numbers and cell motility were examined over a 60 minute period and remained constant when metabolism of substrate was occurring. In cases where no metabolism was observed, the cells lost all motility after a 10 minute incubation period and substantial cell lysis had occurred after 60 minutes of incubation. With glycerol, some loss of cell motility was observed after 60 minutes incubation. This was probably due to inadequate oxygenation of the cell

SUBSTRATE (1mM)	OXIDATION RATE nmol/min/10^8 cells
D-GLUCOSE	25.6
D-MANNOSE	23.4
D-FRUCTOSE	22.9
GLYCEROL	26.6
D-GALACTOSE	0.3
α -GLYCEROL PHOSPHATE	0.2
INOSINE	0.3

Table 2: Table of substrate oxidation rates of 37°C in T. brucei as measured using a Clark oxygen electrode.

solution.

The kinetic parameters of D-glucose metabolism were calculated using the oxygen electrode. The values obtained (Figure 14) were: K_m = 3.22 ± 0.07mM and V_{max} = 62.7 ± 7.5 nmoles/min/10^8 cells. The maximum velocity is within the range measured by Flynn and Bowman (1973) at 37°C.

The kinetic parameters of D-glucose metabolism were also calculated at 20°C to allow comparison with transport assay rates measured at this temperature. The kinetic parameters calculated were K_m = 0.387 ± 0.11mM and V_{max} = 20.3 ± 0.93 nmoles/min/10^8 cells (Figure 15). Both K_m values were similar but increasing the temperature significantly increases the V_{max} for glucose metabolism.

4.1.2: Inhibition of D-glucose oxidation

The inhibition of the metabolism of D-glucose was measured by addition of the appropriate amount of potential inhibitor to the electrode immediately prior to D-glucose addition. The rate of oxygen consumption was compared to that with no inhibitor present. The percentage inhibition by 3-0-methyl-D-glucose and 6-deoxy-D-glucose is shown in Table 3 whilst Figure 16 shows the percentage inhibition of D-glucose oxidation by 40mM 1-deoxy-D-glucose at a range of D-glucose concentrations.

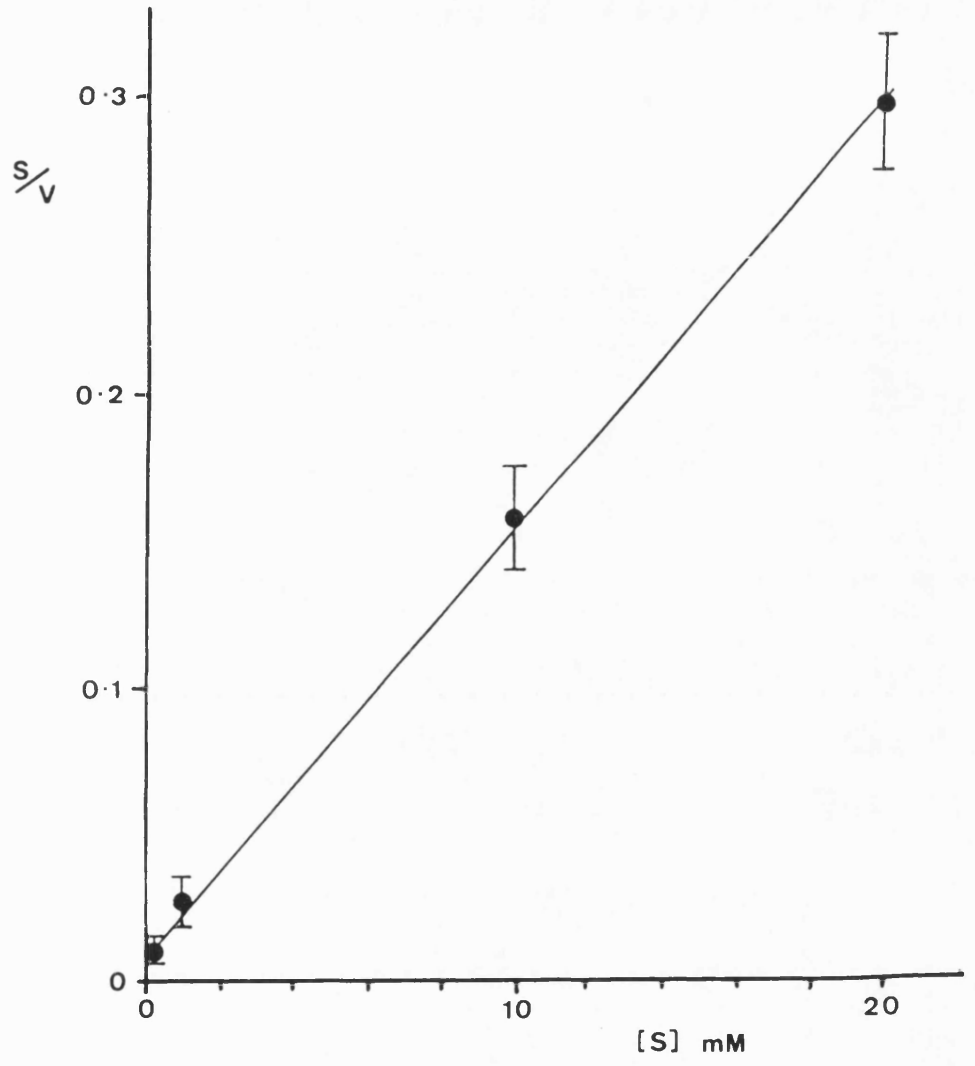


Figure 14: Hanes plot of D-glucose oxidation rate vs. D-glucose concentration at 37°C by T. brucei. K_m = 0.322 ± 0.07mM, V_{max} = 62.7 ± 7.5 nmol/min/10^8 cells, n = 2. Line fitted by method of least squares.

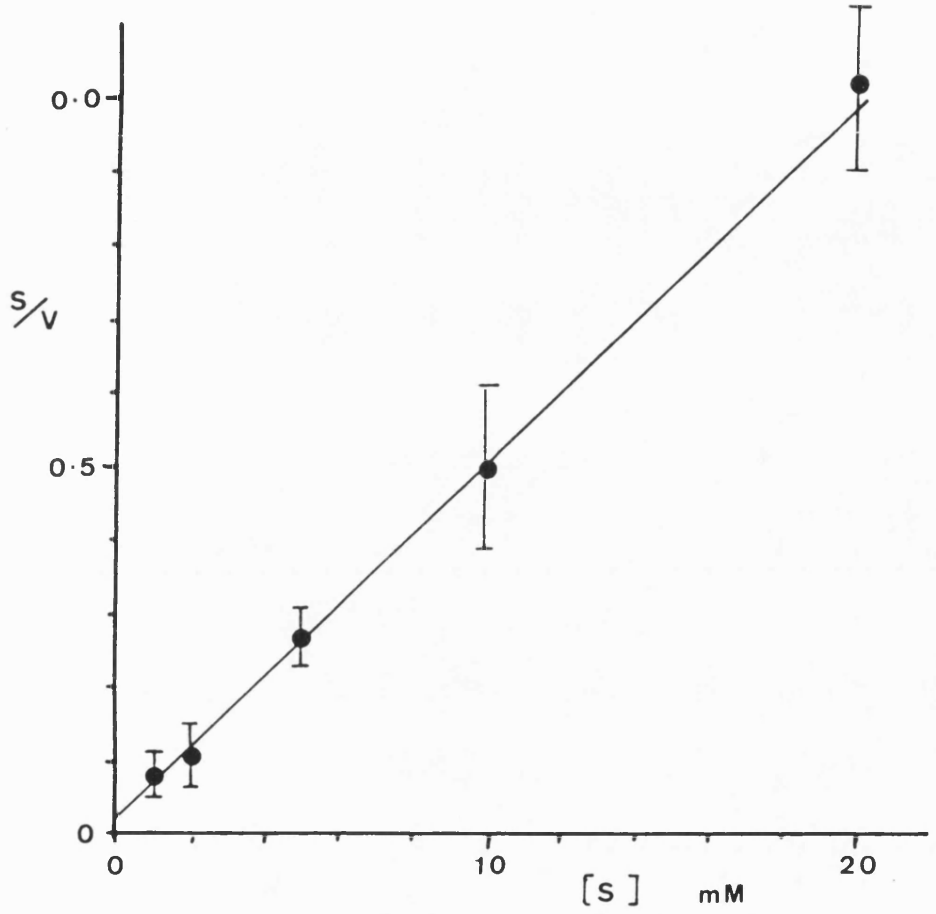


Figure 15: Hanes plot of D-glucose oxidation rate vs. D-glucose concentration at 20°C by T. brucei. K_m = 0.387 ± 0.11mM, V_{max} = 20.3 ± 0.93 nmol/min/10^8 cells, n = 2. Line fitted by method of least squares.

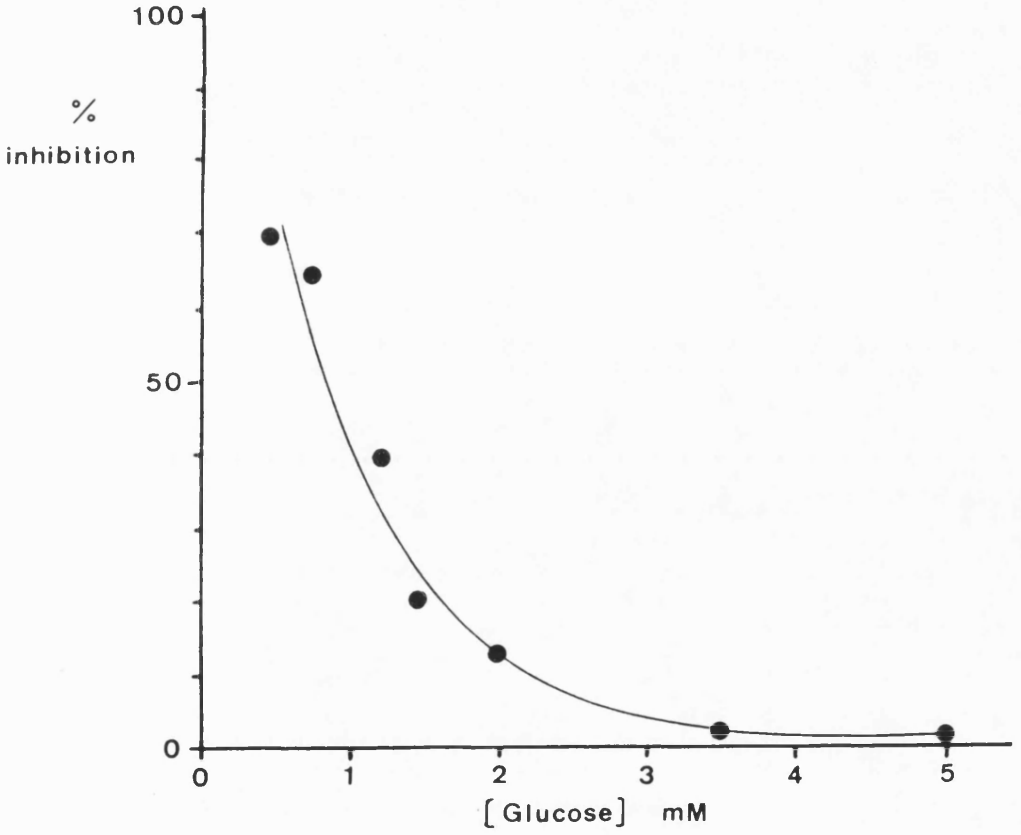


Figure 16: Inhibition of the rate of D-glucose respiration in T. brucei by 40mM 1-deoxy-D-glucose. Percentage inhibition given by 100 x $(1-V_i/V_o)$ where V_i and V_o are the rates of oxygen utilisation in the presence and absence, respectively, of 1-deoxy-D-glucose.

	1mM D-glucose	+ 10mM 6-deoxy D-glucose	+ 10mM 3-0-methyl-D-glucose
Oxidation rate nmol/min/10^8 cells	31.8	1.9	6.6
% Inhibition	-	94.1%	79.3%

Table 3: Inhibition of D-glucose metabolism by 6-deoxy-D-glucose and 3-0-methyl-D-glucose at 37°C.

It was clear from the results that the 3 analogues examined all had an inhibitory effect on substrate metabolism, 6-deoxy-D-glucose being the most potent inhibitor. The analogue 1-deoxy-D-glucose appeared to be the least effective inhibitor with a 40mM substrate concentration causing only 43% inhibition at 1mM substrate concentration compared with 79% inhibition by 10mM 3-0-methyl-D-glucose. The results were, however, from different experiments and direct comparisons may not be applicable as the inhibition by 1-deoxy-D-glucose was measured by addition of inhibitor to cells after a steady state of D-glucose respiration had been reached.

The degree of inhibition of D-glucose metabolism resulted in all the analogues being considered as potential non-metabolised D-glucose analogues. It was necessary to determine that inhibition did occur at the transport stage of D-glucose metabolism and not at a

later stage in the metabolic pathway.

4.2: 1-deoxy-D-glucose transport

Preliminary experiments using 3-0-methyl-D-glucose as a substrate in a transport assay indicated that the analogue was not a suitable substrate as the rate of transport was too slow. The experiments did, however, show that both 6-deoxy-D-glucose and 1-deoxy-D-glucose were effective inhibitors of 3-0-methyl-D-glucose uptake and the availability of radio-labelled 1-deoxy-D-glucose allowed the investigation of its uptake into Trypanosoma brucei.

Preliminary results indicated that uptake occurred at a significantly faster rate than with 3-0-methyl-D-glucose and that measurements of initial rates of uptake in cells starved of metabolisable substrate were feasable before cell death and lysis occurred.

4.2.1: Metabolism of 1-deoxy-D-glucose

It was important to establish whether 1-deoxy-D-glucose was metabolised after uptake had occurred and this was investigated by determining the specificity of the analogue for hexokinase from Trypanosoma brucei.

The assay used was a coupled enzymic assay measuring the production of ADP in the hexokinase-catalyzed reaction. Trypanosomal hexokinase was prepared either via a crude glycosomal preparation or a cell-free extract

(See Methods).

The results (Table 4) indicated that with a fully functioning assay procedure with respect to phosphorylation of D-glucose, the rate of phosphorylation of 1-deoxy-D-glucose was less than 0.1% of the D-glucose phosphorylation rate. No inhibition of the rate of D-glucose phosphorylation by 1-deoxy-D-glucose was detected up to inhibitor to substrate ratios of 40:1. This was also true of 6-deoxy-D-glucose which cannot be phosphorylated and did not inhibit the enzyme.

These results suggested that 1-deoxy-D-glucose may be a suitable non-metabolized analogue for D-glucose transport in T. brucei.

4.2.2: 1-deoxy-D-glucose kinetic experiments

Figure 26a shows a time-course of 1-deoxy-D-glucose uptake. The sugar appeared to reach equilibrium after approximately two minutes. Beyond this time, trapped label was rapidly lost from the cells, this being initially interpreted as onset of cell lysis after depletion of metabolic substrate.

The internal volume accessible to the analogue was calculated from the equilibrium value of the time-course and others like it. The value obtained was $5.9\mu l/10^8$ cells. This value is higher than that calculated by Voorheis and Martin (1980) but less than the value calculated by Damper and Patten (1976).

Subsequent kinetic analysis produced some highly

	ASSAY CONDITIONS	HEXOKINASE ACTIVITY µmoles/min/ml cell preparation
Cell Lysate	Background oxidase activity	0.004
	1-deoxy-D-glucose (1mM)	0.004
	D-glucose (1mM)	0.860 ± 0.025 *
Glycosomal preparation	Background oxidase activity	0.008
	1mM 1-deoxy-D-glucose	0.008
	1mM D-glucose	0.94 ± 0.03 *
	250µM D-glucose	0.52 ± 0.03
	250µM D-glucose + 10mM 1-deoxy-D-glucose	0.55 ± 0.02
	250µM D-glucose + 10mM 6-deoxy-D-glucose	0.51 ± 0.02

Table 4: The activity of hexokinase with 1-deoxy-D-glucose, 6-deoxy-D-glucose and D-glucose. The activity was measured using a coupled enzyme assay measuring the formation of NAD^+. The hexokinase was present in a crude cell lysate or a glycosomal preparation prepared from T. brucei.
* ± range, n = 2.

anomalous results. The zero-trans entry kinetic parameters were: K_m = 4.03 ± 0.42mM and V_{max} = 0.052 ± 0.005mM sec^{-1} (Game et al., 1986), the maximum velocity being particularly slow for such a rapidly metabolising organism.

Attempts to measure efflux indicated that the rate was much slower than that of uptake and inhibitor experiments produced peculiar biphasic inhibition profiles suggesting two sites of inhibition. It was clear that either the transport system for 1-deoxy-D-glucose was not a straightforward facilitated diffusion system, or the methodology used was inadequate for correct measurement of uptake.

To further investigate the transport process, a counter-flow experiment was carried out preincubating the cells with 10mM 1-deoxy-D-glucose for 10 minutes prior to addition of labelled analogue. The results shown in Figure 17 indicated that there was little difference in the uptake profile of the counter-flow time-course and the zero-trans time-course. This suggested that in the zero-trans experiment, the label was being accumulated above the value expected at equilibrium and the subsequent efflux of label was not due to cell lysis but to the loss of the agent or process causing the initial accumulation. This would mean that the time-course of 1-deoxy-D-glucose uptake was wrongly interpreted as uptake until equilibrium had been reached and the true eqilibrium value was thus lower than that previously

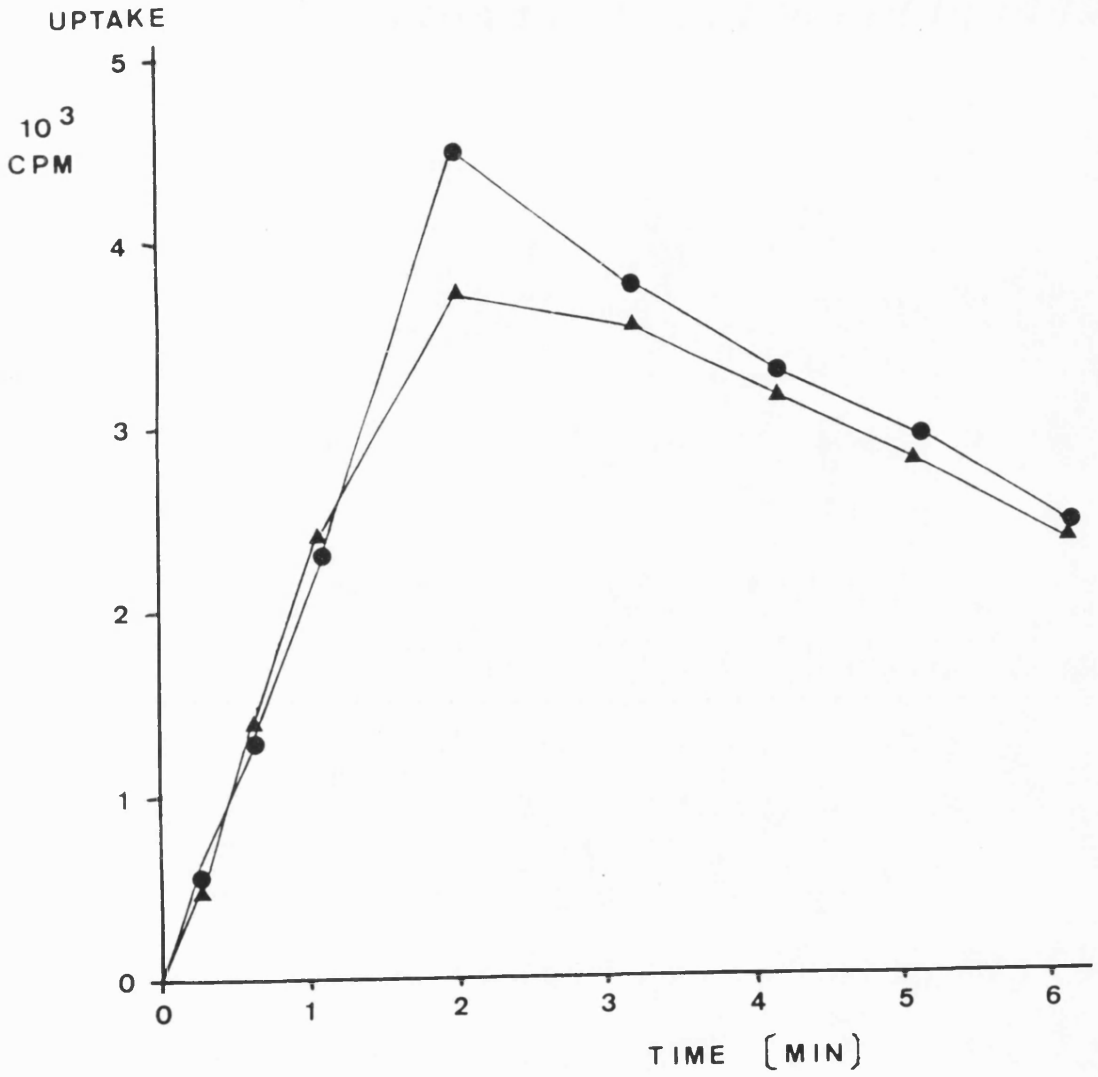


Figure 17: Uptake of 3µM 1-deoxy-D-glucose after 10 minutes preincubation with 10mM unlabelled 1-deoxy-D-glucose (●), no preincubation (▲).

calculated.

A subsequent experiment (Figure 18) indicated that efflux of 1-deoxy-D-glucose from preloaded cells was accelerated by the addition of D-glucose externally. This suggested a possible explanation of the uptake profile in that the internal D-glucose concentration which was thought to be very low inside the cells was in fact high enough to cause counter-flow and thus accumulation of 1-deoxy-D-glucose during uptake. This would, however, mean that transport was not rate-limiting.

The above hypothesis was investigated by attempting to measure the internal D-glucose concentration using the glucose oxidase/peroxidase assay. The result of the assay was that no D-glucose could be detected internally, the internal concentraton being at least 20 times lower than the external D-glucose concentration. Such a low concentration could not be responsible for the accumulation of 1-deoxy-D-glucose by counter-flow thus disproving the above hypothesis.

4.2.3: Energy requirements for transport

The probable accumulation of 1-deoxy-D-glucose suggested that there may be an energy requirement for the transport process. To investigate this possibility the following experiment was devised:

Cells were preincubated at 37^{o}C for sufficient time to deplete the level of ATP. A 3 minute preincubation

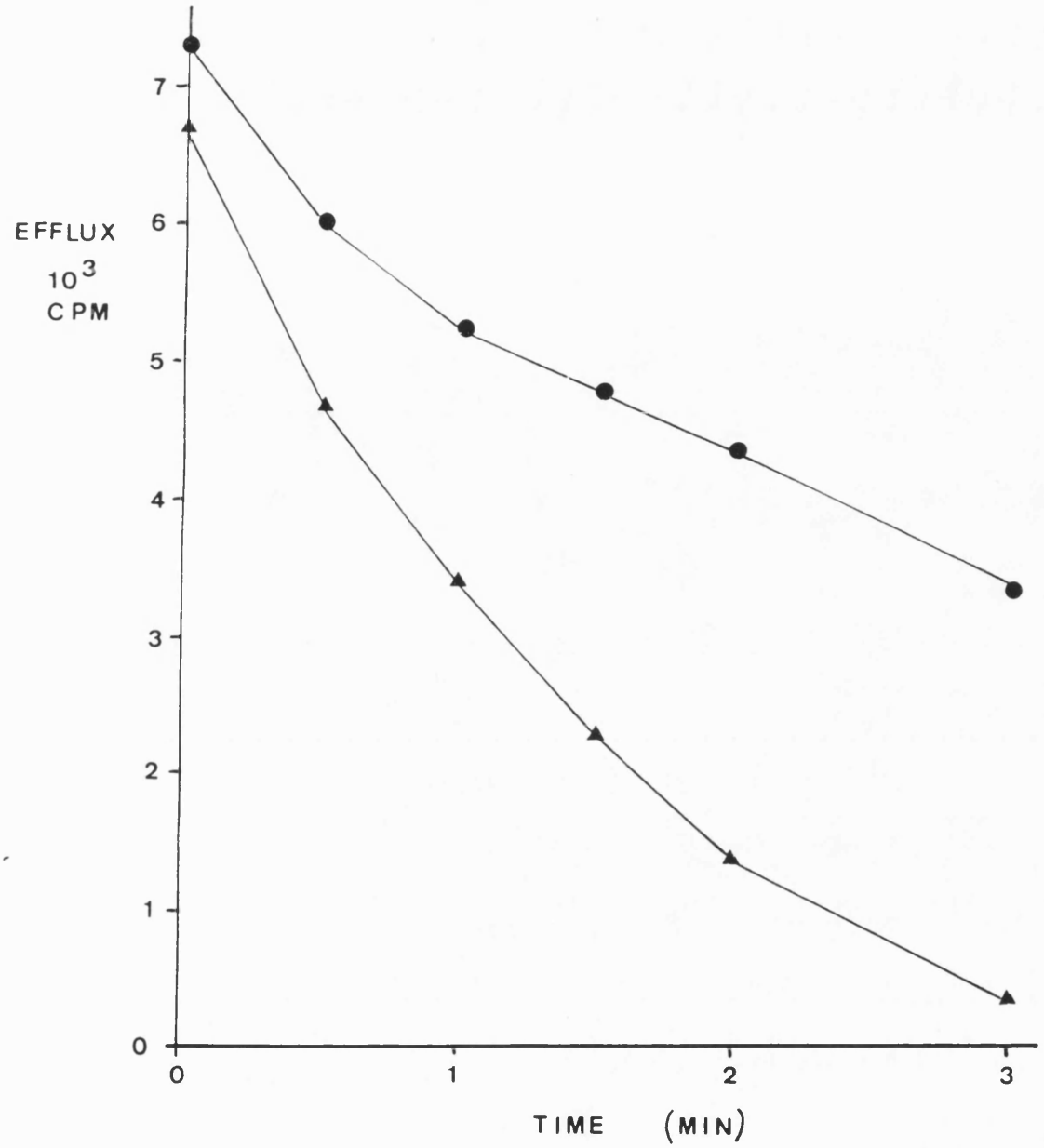


Figure 18: Efflux of 50μM 1-deoxy-D-glucose vs. time at 37°C (●). Efflux of 50μm 1-deoxy-D-glucose vs time with 2mM D-glucose in the external medium at 37°C (▲).

time was found to be required to produce the maximum effect. This period of time corresponded to the time taken to accumulate the maximum label in the normal uptake time-course. After preincubation, the rate of uptake was measured. The results (Figure 19) indicated that the depletion of ATP caused an 11-fold drop in the initial rate of uptake. Also if D-glucose was reintroduced after preincubation and the cells allowed to recover for 10 minutes, the initial rate of transport of 1-deoxy-D-glucose was largely restored to previous zero-trans levels. This experiment suggested that either energy is required for the major component of transport to occur, or that metabolism of the label was occurring and thus trapping the label in a reversible fashion. It was considered a possibility that the transport occurring after the preincubation period reflected the true non-metabolised rate. This was further investigated but it was found that the transport was not saturable by high concentrations of cold analogue nor inhibited by 6-deoxy- or 2-deoxy-D-glucose.

Other analogues were investigated in terms of transport occurring before and after 3 minute preincubation periods. Figure 20 represents the time-courses of 3-O-methyl-D-glucose, 2-deoxy-D-glucose and D-galactose before and after preincubation. The figures show that both 3-O-methyl-D-glucose and 2-deoxy-D-glucose had reduced rates of uptake after preincubation, but D-galactose did not demonstrate such a change. As D-galactose is not metabolised and very probably not transported, the uptake

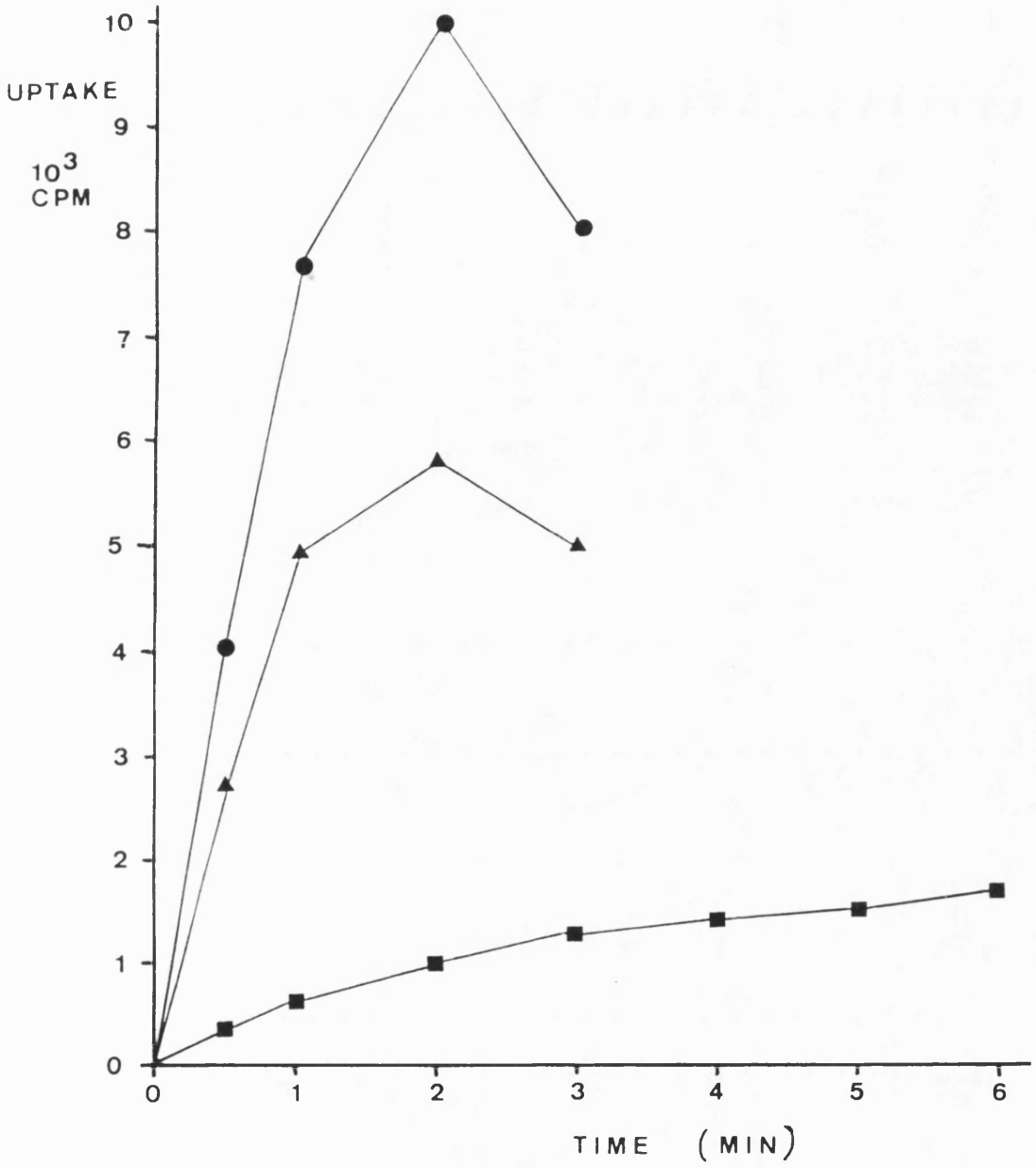


Figure 19: Uptake of 10μM 1-deoxy-D-glucose at 37°C vs. time after no preincubation (●), 3 minutes preincubation in buffer at 37°C (■), 3 minutes preincubation at 37°C followed by reintroduction of 10mM D-glucose for 10 minutes (▲).

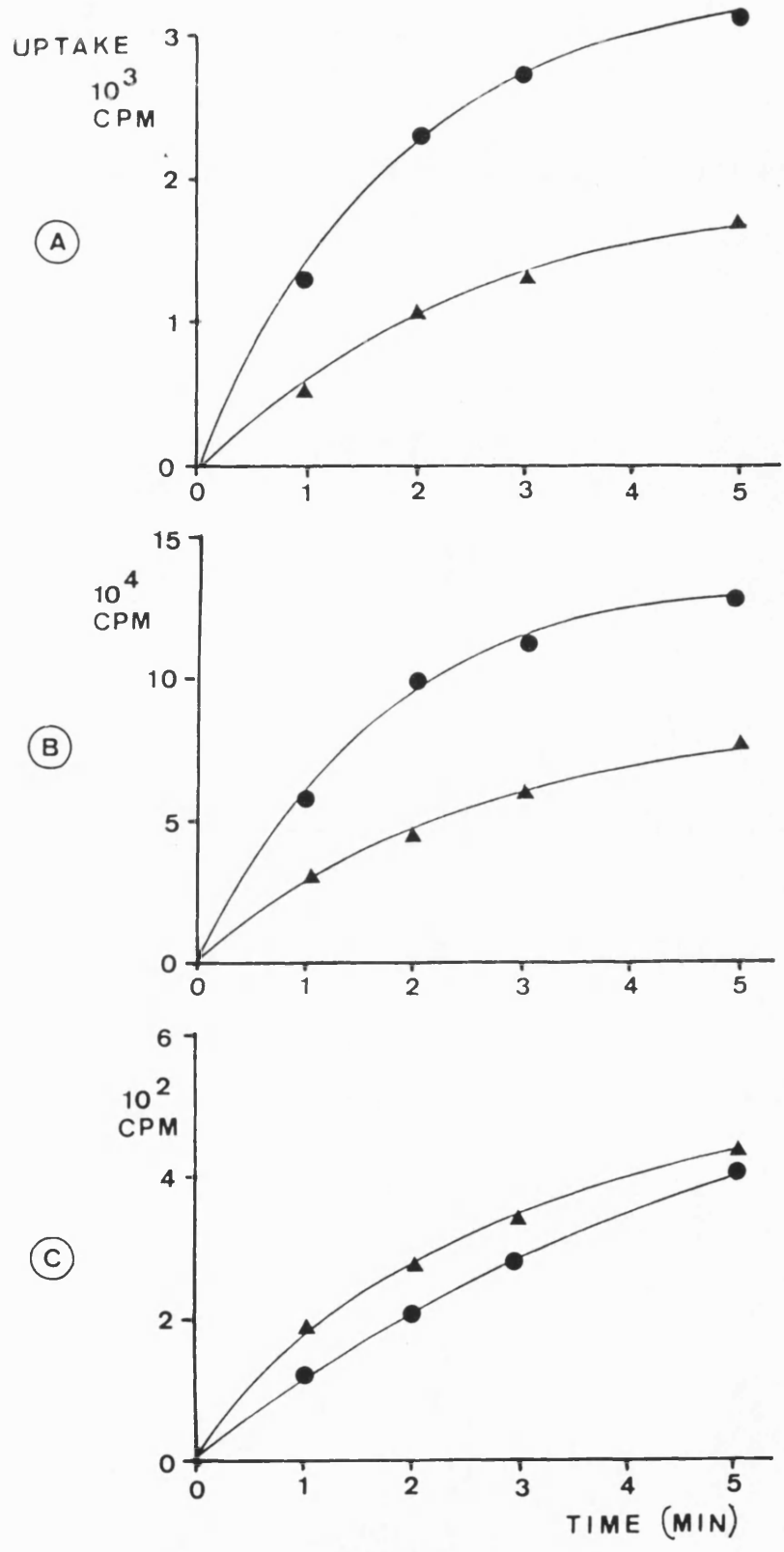


Figure 20: Uptake of A) ^{14}C-3-O-methyl-D-glucose, B) ^{3}H-2-deoxy-D-glucose, C) ^{14}C-D-galactose after 3 minutes preincubation (▲), or with no preincubation (●). All radio-labels were at 'tracer' concentrations.

occurring after preincubation was concluded to be non-specific leakage due to the loss of cell integrity. This was also reflected in the low numbers of counts trapped when incubating the cells with radio-labelled D-galactose.

4.2.4: Thin layer chromatographic analysis of transported analogues

To investigate the possibility of a group-translocase system being responsible for transport and subsequent phosphorylation of the analogue, a means of analysis of the transported analogue using thin layer chromatography (TLC) to measure the mobility of the sugar was devised (See Methods). Both labelled 1-deoxy-D-glucose and 2-deoxy-D-glucose were incubated with cells and the resulting sugar extracts spotted onto silica-gel plates. These were then developed in butanol:ethanol:water, (52:33:15). It was apparent from the results (Figure 21) that almost all transported label was altered and the use of standard sugars revealed the altered compounds to be the phosphorylated sugar derivative.

Previous time-courses had shown a period of efflux alter the peak of trapped label had been reached, so a further experiment was devised to determine whether the trapped phosphorylated sugar was dephosphorylated prior to or during efflux, or that the efflux represented leakage of phosphorylated sugar due to loss of cell integrity. The experiment involved incubating cells

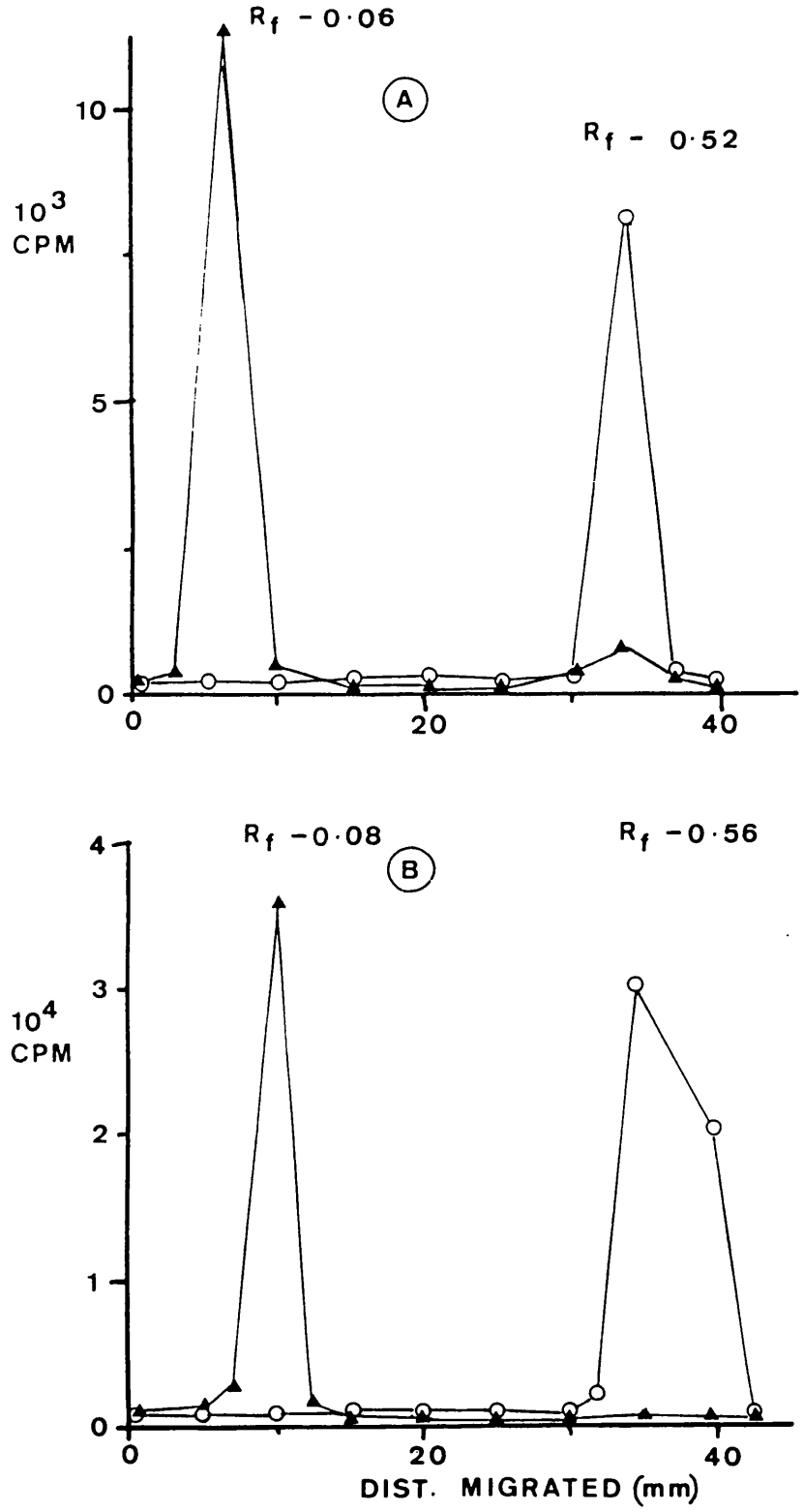


Figure 21: Mobility of A) 1-deoxy-D-glucose, B) 2-deoxy-D-glucose on TLC plates before uptake - (O) and after uptake - (▲). TLC plates developed in Butanol:ethanol:water, (52:33:15).

with labelled 1-deoxy-D-glucose for 30 seconds, washing the cells to remove all extracellular label, then resuspending the cells in buffer at 37°C and taking samples at various time points up to 10 minutes. The samples were centrifuged and the supernatant retained. The pellet was washed twice and both the pellet and supernatant extracted for subsequent TLC analysis. Figure 22 demonstrates clearly that the label within the cells was entirely in the phosphorylated form and its decrease during efflux was mirrored by an increase in free sugar outside the cells. No phosphorylated sugar was present outside the cells. From these results it was clear that the time-course for uptake represented a period of accumulation of phosphorylated 1-deoxy-D-glucose which continued until the source of the phosphate was depleted. The time taken to reach the peak sugar phosphate labels was similar to that of the loss of motility of the cells in the absence of substrate. The source of high-energy phosphate was assumed to be ATP.

As the rate of 1-deoxy-D-glucose efflux was slower than uptake, it was considered unlikely that dephosphorylation was occurring during efflux, but rather due to the action of a trypanosomal phosphatase activity.

The results from the hexokinase assay, indicating 1-deoxy-D-glucose not to be a substrate for T. brucei hexokinase, suggested that the phosphorylation may have been carried out by some other means. It was however, clear that 1-deoxy-D-glucose was not a suitable substrate

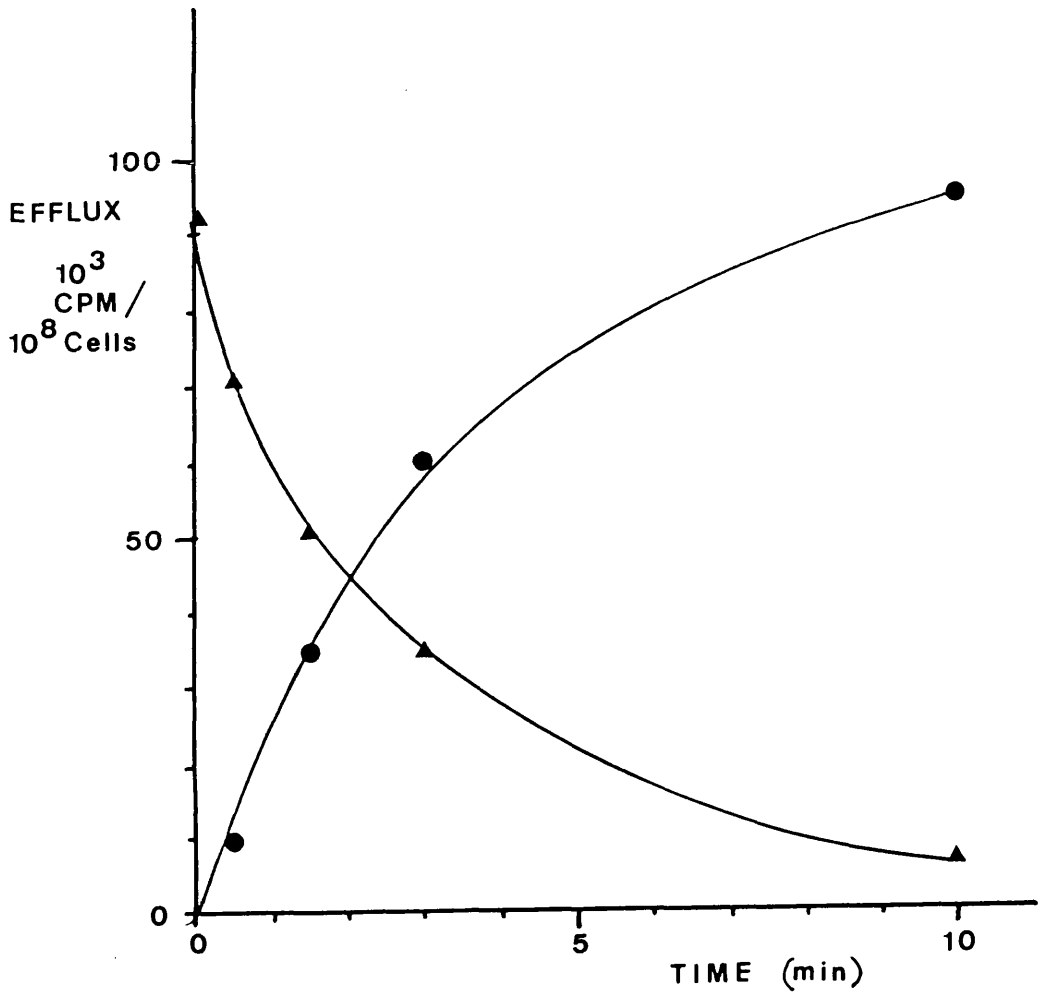


Figure 22: Efflux of 500μM 1-deoxy-D-glucose vs. time at 37°C. The figure shows phosphorylated sugar remaining inside cells (▲) and free sugar appearance outside cells (●).

for the kinetic analysis of sugar transport due to the high rate of phosphorylation occurring. The results with 1-deoxy-D-glucose indicated that a truly non-metabolised analogue would be necessary to determine whether transport occurred prior to any subsequent metabolism.

It was at this stage that a concerted effort was made to synthesise labelled 6-deoxy-D-glucose (See Chapter 3). The eventual success of this effort indicated the need for improvements in the methodology of the uptake procedure when using 6-deoxy-D-glucose as a substrate for transport (See Chapter 4.3).

The resultant much reduced incubation time, lower temperature, and the greater effectiveness of the stopping solution was utilised when repeating experiments described in this section which analysed the state of transported analogues.

The results in Figure 23 indicate that, after a 3 second incubation time, no 6-deoxy-D-glucose had been metabolised. Also, after transport of 1-deoxy-D-glucose, a significant proportion of the transported label was in the phosphorylated form. The high degree of phosphorylated sugar present after only 3 seconds incubation suggests a very rapid rate of phosphorylation by normally-metabolising trypanosomes. Further experiments were carried out where trypanosomes were preincubated for 10 and 20 minutes at room temperature to deplete the ATP levels in the cells. The proportion of transported sugar phosphorylated was then investigated using TLC.

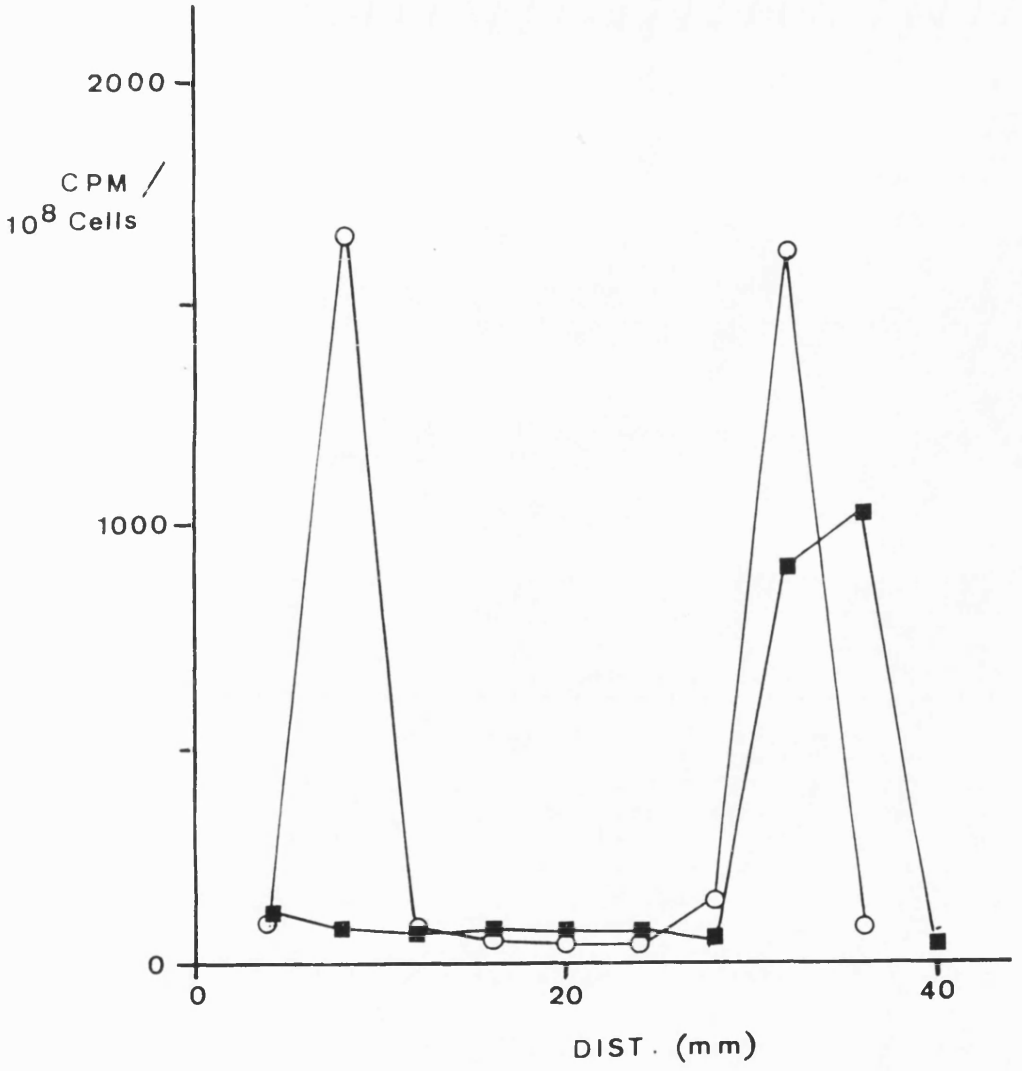


Figure 23: TLC analysis of 3 second uptake time-points of 1-deoxy-D-glucose (○) and 6-deoxy-D-glucose (■) at 20°C. Peaks at 8mm correspond to sugar phosphate. TLC plates were developed in Butanol:ethanol:water, (52:33:15).

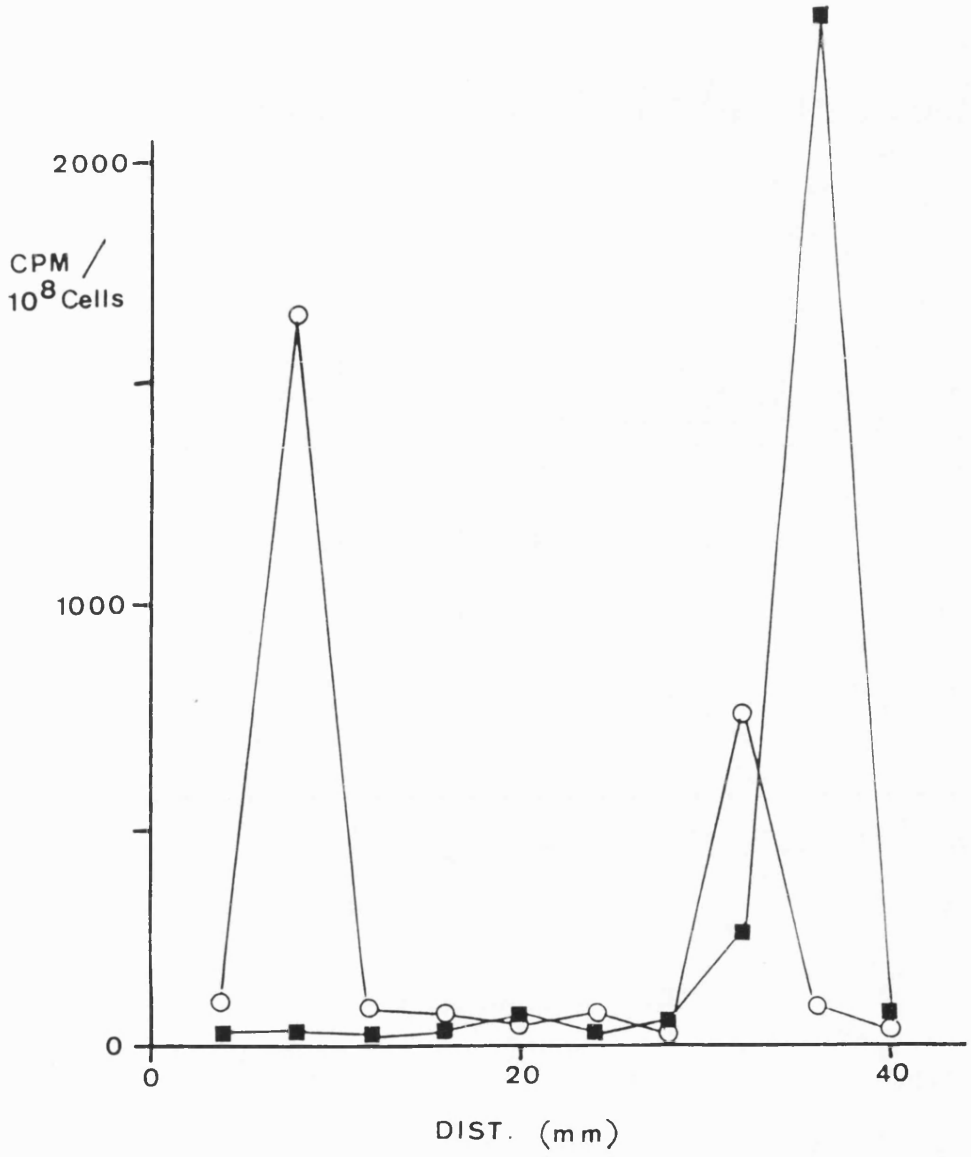


Figure 24: TLC analysis of 3 second uptake time-points of 1-deoxy-D-glucose (○) and 6-deoxy-D-glucose (■) at 20°C after 10 minutes preincubation without substrate. TLC plates were developed in Butanol:ethanol:water, (52:33:15).

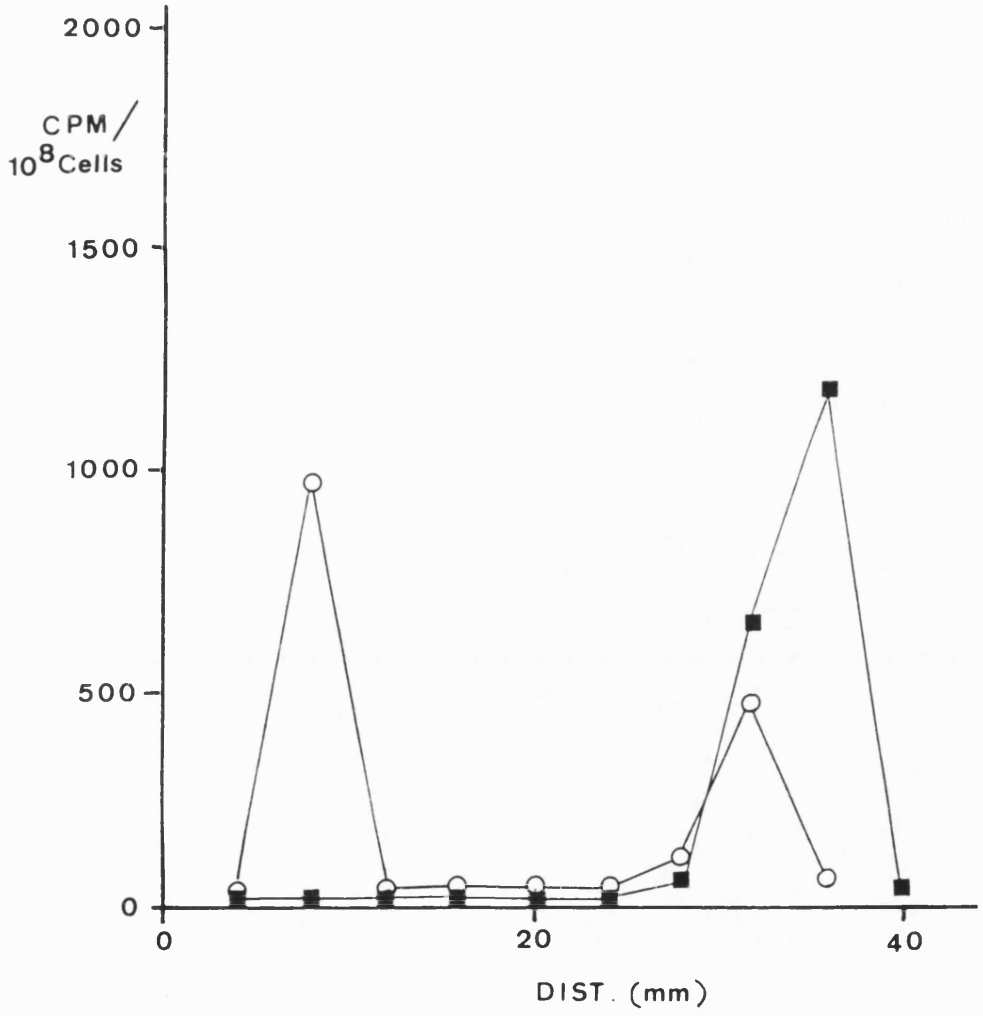


Figure 25: TLC analysis of 3 second time-points of 1-deoxy-D-glucose (○) and 6-deoxy-D-glucose (■) at 20°C after 20 minutes preincubation without substrate. TLC plates were developed in Butanol:ethanol:water, (52:33:15).

The results (Figures 24 and 25) indicate that despite the depletion of ATP levels (no motility of trypanosomes) a high proportion of the transported 1-deoxy-D-glucose was phosphorylated during a 3 second incubation period. This may have been indicative of an alternative reservoir of transferable phosphate. The transport of 6-deoxy-D-glucose continued after the preincubation periods with no visible metabolism occurring although the total CPM transported decreased after 20 minutes preincubation in the absence of substrate. This probably represents a degree of energy starvation sufficient to deplete the population of viable cells despite the phosphorylation of transported 1-deoxy-D-glucose.

4.2.5: 1-deoxy-glucose kinetic parameters

The changes made to the uptake assay procedure made possible a more accurate determination of the zero-trans entry kinetic parameters for 1-deoxy-D-glucose. Figure 26b indicated that a time-course of 100μM 1-deoxy-D-glucose did not follow saturation kinetics over a 60 second time interval. Using the value for the internal volume of T. brucei cells accessible to analogue (Chapter 4.3.2), it can be seen that the accumulation of label is considerably above the expected equilibrium value for a non-metabolised analogue. The early part of the time-course did however, approximate to a normal uptake profile and the error in reduced backflux due to trapped phosphorylated analogue was considered acceptably small.

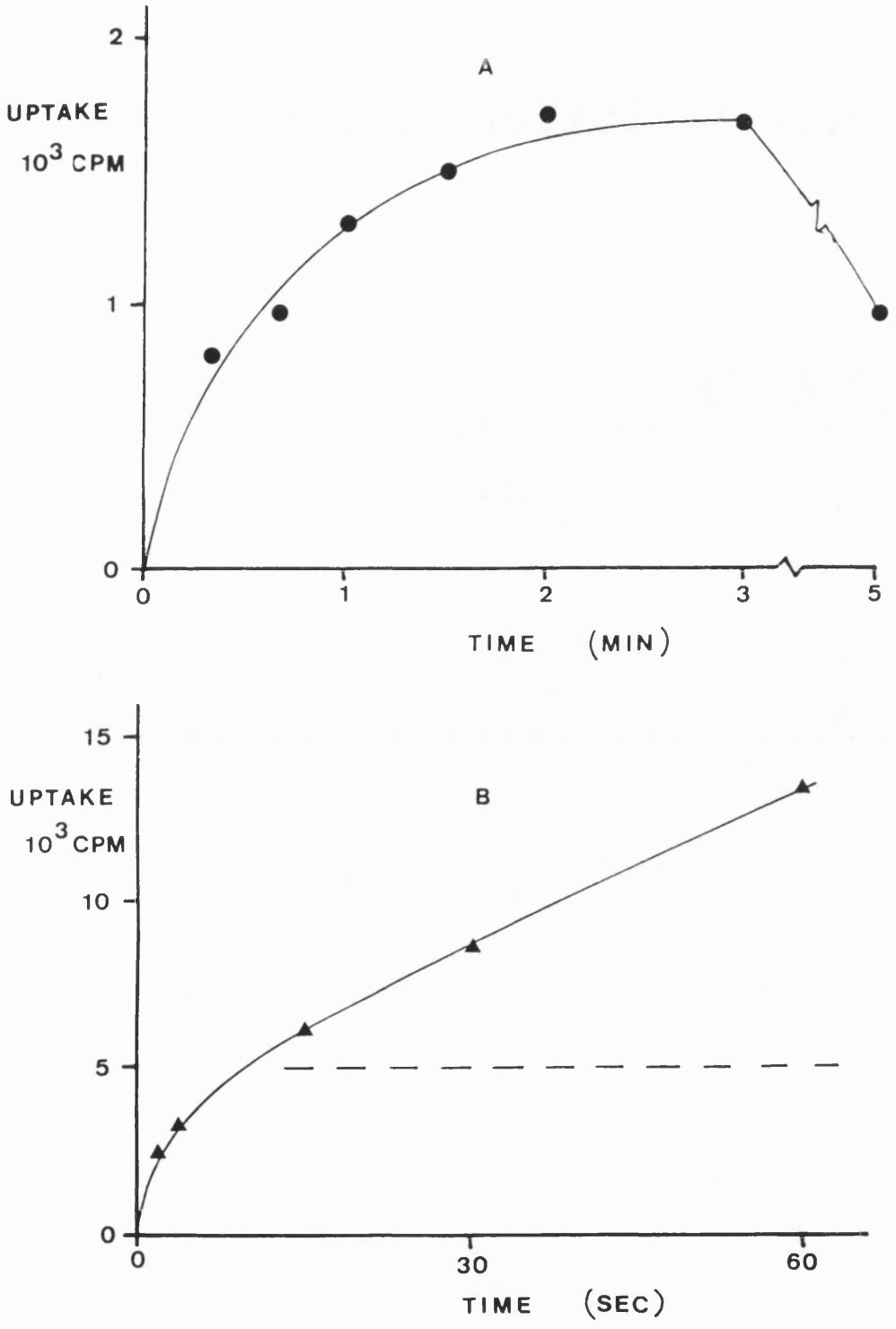


Figure 26: Uptake of 100μM 1-deoxy-D-glucose vs. time. A) at 37°C using the 1-deoxy-D-glucose uptake protocol (●), B) at 20°C using the later 6-deoxy-D-glucose uptake protocol (▲). The broken line represents the calculated equilibrium value from 6-deoxy-D-glucose uptake. Uptake by both methods used 1μl of radio-label per 5×10^7 cells.

A single time point assay using an incubation time of 3 seconds was used to calculate the zero-trans entry kinetic parameters as shown in Figure 27. The K_m value of 3.41 ± 0.262mM for 1-deoxy-D-glucose uptake was exactly the same as the K_i for 1-deoxy-D-glucose inhibition of 6-deoxy-D-glucose uptake (See Chapter 4.4.2). The V_{max} of 0.323 ± 0.016mM sec^{-1} was also similar to the V_{max} under zero-trans entry conditions for 6-deoxy-D-glucose uptake (See Table 6). The similarity between the kinetic parameters of the two analogues suggests that both analogues use the same transport site.

Due to the high level of metabolism of 1-deoxy-D-glucose, it was not possible to use other standard kinetic approaches for the complete kinetic analysis of the T. brucei hexose transporter.

4.2.6: Inhibitors of 1-deoxy-D-glucose uptake

To further establish that only one transport pathway for both 1-deoxy-D-glucose and 6-deoxy-D-glucose was in operation in Trypanosoma brucei, simple percentage inhibition studies using a range of D-glucose analogues, were performed with both 1-deoxy-D-glucose and 6-deoxy-D-glucose as substrates for uptake. The results shown in Table 5 indicate the percentage inhibition, by a 20mM concentration of inhibitor, of the uptake of 100μM substrate after 3 seconds incubation at 20°C. From these results an approximate indication of the specificity

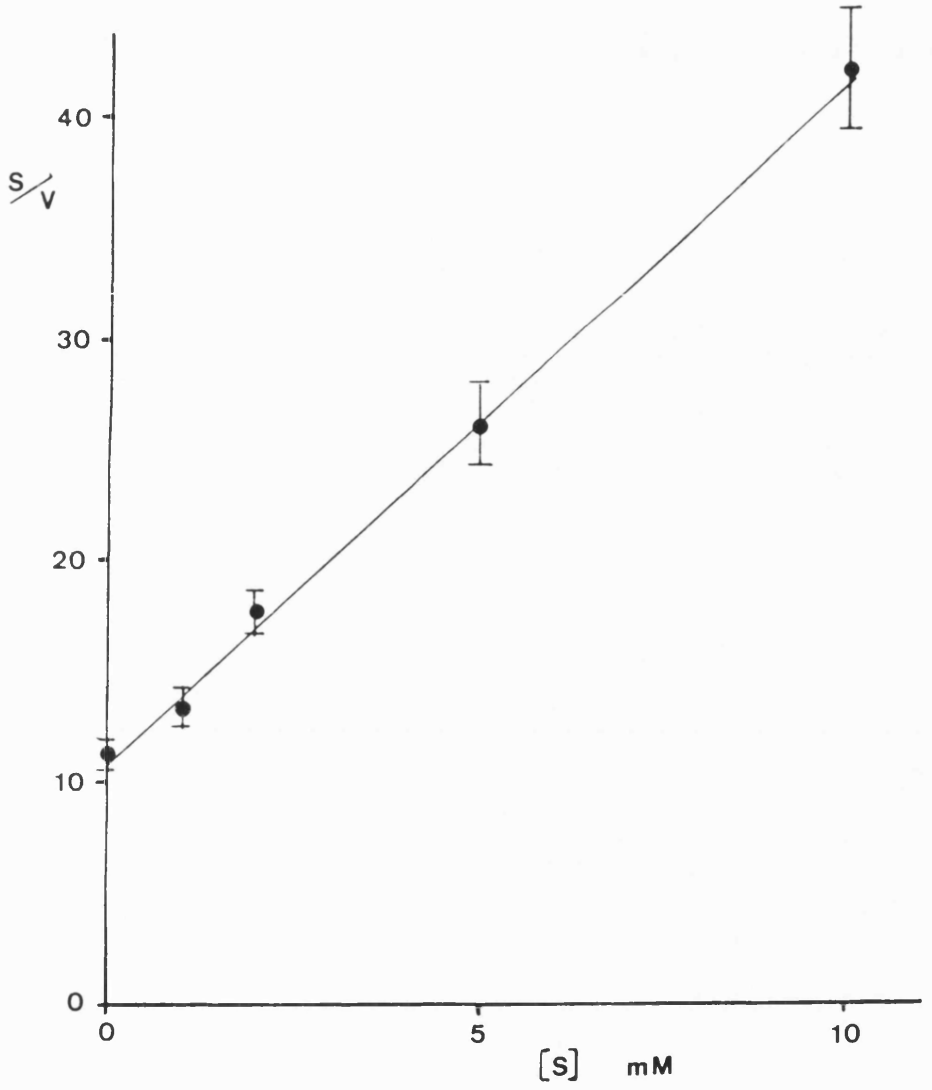


Figure 27: Hanes plot of 1-deoxy-D-glucose uptake under zero-trans conditions using a 3 second single time-point assay at 20°C. K_m = 3.41 ± 0.262 mM, V_{max} = 0.323 ± 0.016mM/sec, n = 4. Line fitted by method of least squares.

20mM INHIBITOR	PERCENTAGE INHIBITION	
	100μM 1-DEOXY-D-GLUCOSE	100μM 6-DEOXY-D-GLUCOSE
D-glucose	88.2	87.7
D-fructose	72.2	68.4
D-mannose	91.75	90.4
Glycerol	11.75	7.0
α-methyl-D-glucosdide	0.0	0.0
1-deoxy-D-glucose	87.5	86.3
2-deoxy-D-glucose	96.3	96.2
2-fluoro-2-deoxy-D-glucose	99.5	96.9
D-glucosamine	62.3	45.4
N-acetyl-D-glucosamine	73.7	60.9
3-O-methyl-D-glucose	66.9	60.3
3-fluoro-3-deoxy-D-glucose	99.0	97.5
5-thio-D-glucose	65.8	48.7
6-deoxy-D-glucose	86.9	92.0
6-chloro-6-deoxy-D-glucose	95.4	95.2

Table 5: Percentage inhibition of 100μM 6-deoxy-D-glucose and 1-deoxy-D-glucose by a range of D-glucose analogues. Inhibition was measured using zero-trans conditions in a single time-point (3 second) uptake assay at 20°C.

of binding to the sugar transport site was obtained. There were no significant differences in the specificity of inhibition of 1-deoxy-D-glucose and 6-deoxy-D-glucose uptake. This result provides further evidence that both glucose analogues use the same transporter and this site is used by the normal substrates for T. brucei metabolism with the exception of glycerol.

4.3: 6-deoxy-D-glucose kinetic analysis

4.3.1: Transport assay conditions

When labelled 6-deoxy-D-glucose became available for transport experiments, initial studies were performed to determine whether transport of the analogue was occurring and whether the assay procedure previously used was suitable. Initial results indicated that transport of 6-deoxy-D-glucose by intact trypanosomes was occurring but the assay procedure, as used with initial 1-deoxy-D-glucose experiments, was not working sufficiently well to effectively trap transported label.

Time-course experiments indicated that equilibrium was reached much more rapidly by 6-deoxy-D-glucose but inhibition by saturating concentrations of unlabelled 6-deoxy-D-glucose did not occur. It was concluded that the use of 5 volumes of ice-cold buffer as a stopper was not sufficient to prevent efflux of the non-metabolised transported label during the wash procedure.

The use of the known inhibitor phloridzin (Gruenberg

et al., 1978) as a potential stopping agent in conjunction with 20 volumes of ice-cold buffer was investigated and was found to be much more effective. A concentration of 2.2mM (0,1g/100ml) phloridzin was used as this concentration was stable in cold solution and effective as a stopper. Higher concentrations of up to 5mM were investigated but considerable heating of the buffer was required to dissolve the phloridzin and some recrystallisation occurred at 2°C. The higher concentrations of phloridzin also affected the pelleted trypanosomes rendering suspension much more difficult.

The final changes in the assay procedure were:

1. The change of stopper conditions and the subsequent reduction in volume of the reaction mixture. This allowed addition of stopper in an appropriately-sized reaction vessel.
2. Reduction of the assay temperature to 20°C from 37°C.
3. An increase in the amount of labelled 6-deoxy-D-glucose used to compensate for the lower counts per minute at true equilibrium values.
4. A decrease in the assay incubation times when estimating initial rates of uptake due to the high rate of hexose transport.

With the parameters of the assay procedure determined, the effectiveness of the stopper containing phloridzin was compared with the use of cold buffer alone.

Trypanosomes were preloaded with 100μM labelled 6-deoxy-D-glucose until equilibrium was reached, then stopped by the addition of either 20 volumes of ice-cold buffer containing phloridzin, or 5 volumes of ice-cold buffer. After the appropriate incubation time the cells were processed as described in the protocol. The cells stopped with 5 volumes of buffer only, were further diluted with 15 volumes of ice-cold buffer containing an appropriate concentration of phloridzin to be equivalent to the normal stopper conditions.

The results shown in Figure 28 clearly indicate that the previous conditions used to stop uptake were insufficient to prevent leakage of trapped label even at temperatures of 0-2^{o}C. The stopper containing phloridzin did prevent efflux of trapped label over the period of the time-course.

The processing of cells after incubation was stopped required a time of approximately 90 seconds which was well within the period of stability of the cells as indicated in Figure 28.

4.3.2: Determination of the intracellular water space

The improved uptake protocol and the use of a truly non-metabolised glucose analogue allowed the calculation of the intracellular water space for T. brucei from a time-course of 6-deoxy-D-glucose uptake. Such a time-course is shown in Figure 29. The result indicated that using 100μM 6-deoxy-D-glucose, uptake was very rapid with

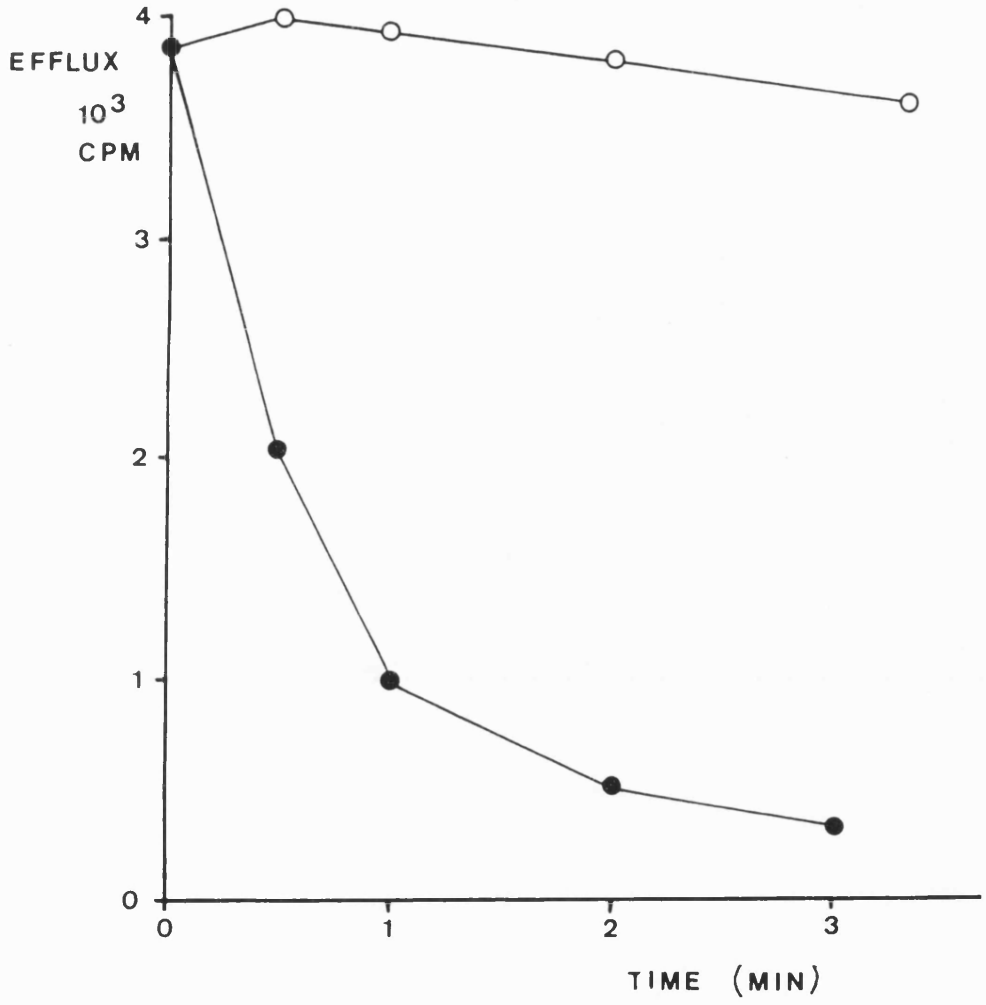


Figure 28: Efflux of 100μM 6-deoxy-D-glucose from preloaded cells 'stopped' by addition of 5 volumes of ice-cold buffer (●), or 20 volumes of ice-cold buffer containing 1% phloridzin (2.2mM) (○).

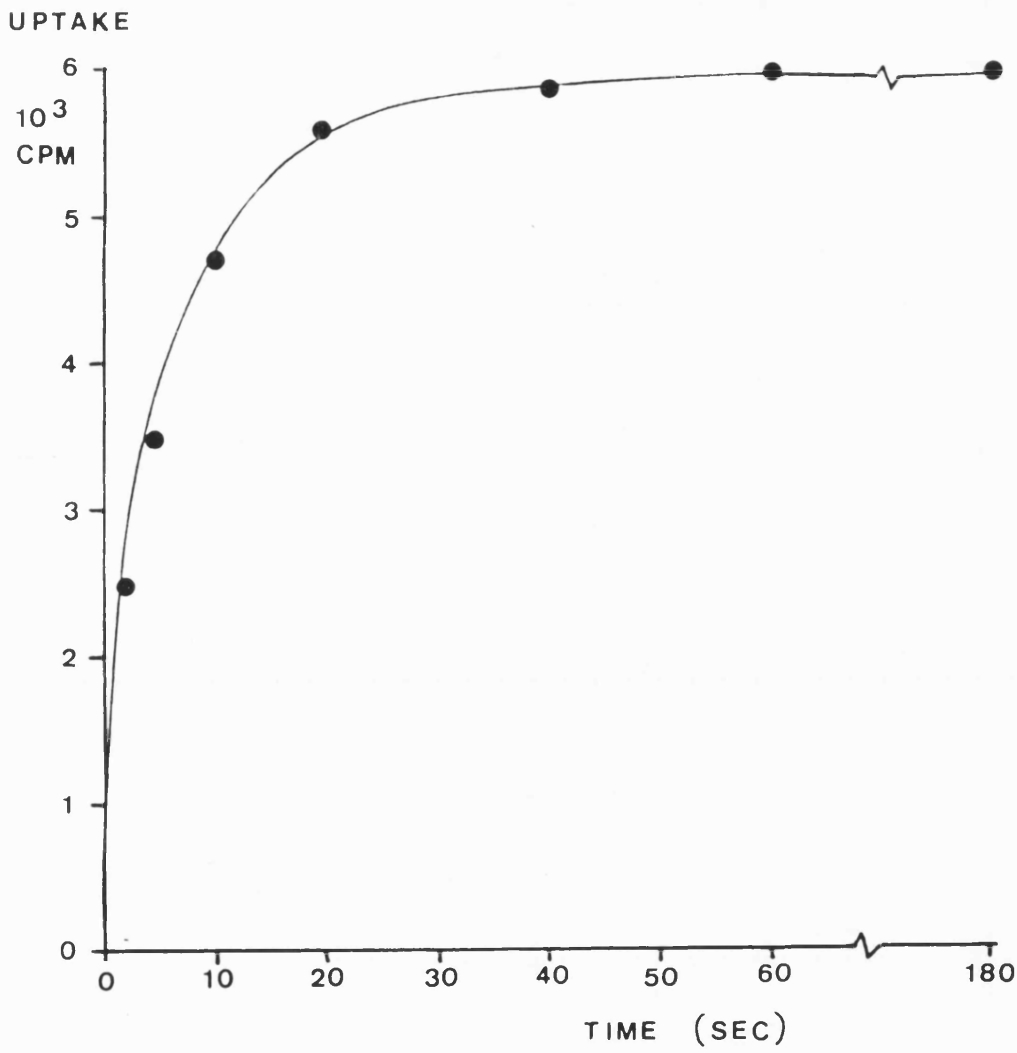


Figure 29: Time-course for uptake of 100μM 6-deoxy-D-glucose vs time at 20°C.

a '$t_{\frac{1}{2}}$' of approximately 4 seconds. Equilibrium was reached after about 30 seconds and remained stable for several minutes.

Using the number of counts trapped at equilibrium and the specific radio-activity of the substrate, the intracellular volume accessible to the analogue was calculated as 1.19 ± 0.13µl per 10^8 cells (See Figure 30). The value is comparable to that of 1.7µl per 10^8 cells calculated by Voorheis and Martin (1980), but is much less than the value of 9.5µl per 10^8 cells determined by Damper and Patten (1976) who calculated the volume of liquid displaced by the total pellet of the cells.

The use of a non-transported labelled marker to determine the proportion of counts in the extracellular space of the pellet was not considered necessary as the counts in the zero-time determinations were very low due to the large relative volume of the stopping solution used to wash the cells.

4.3.3: Zero-trans entry experiments

The determination of kinetic constants for zero-trans entry experiments was initially carried out using a single time-point assay at each appropriate concentration. The time-point used was 4 seconds as this gave a reasonable estimate of the initial rate of uptake. The results obtained were K_m = 2.16 ± 0.068mM, V_{max} = 0.27 ± 0.006mM sec^{-1} (Figure 31).

Concentration of 6-deoxy-D-glucose	=	100µM
Total radio-activity added to the assay (10µl of 1mM 6-deoxy-D-glucose)	=	1,187,335CPM
Total volume of the assay	=	100µl

Therefore:

Counts per minute per µl of extracellular volume	=	11,873CPM
At equilibrium, uptake by 10^8 cells	=	14,998CPM
Internal volume of 10^8 cells	=	1.26µl

Figure 30: Example of a calculation of the intracellular space accessible to 100µM 6-deoxy-D-glucose.

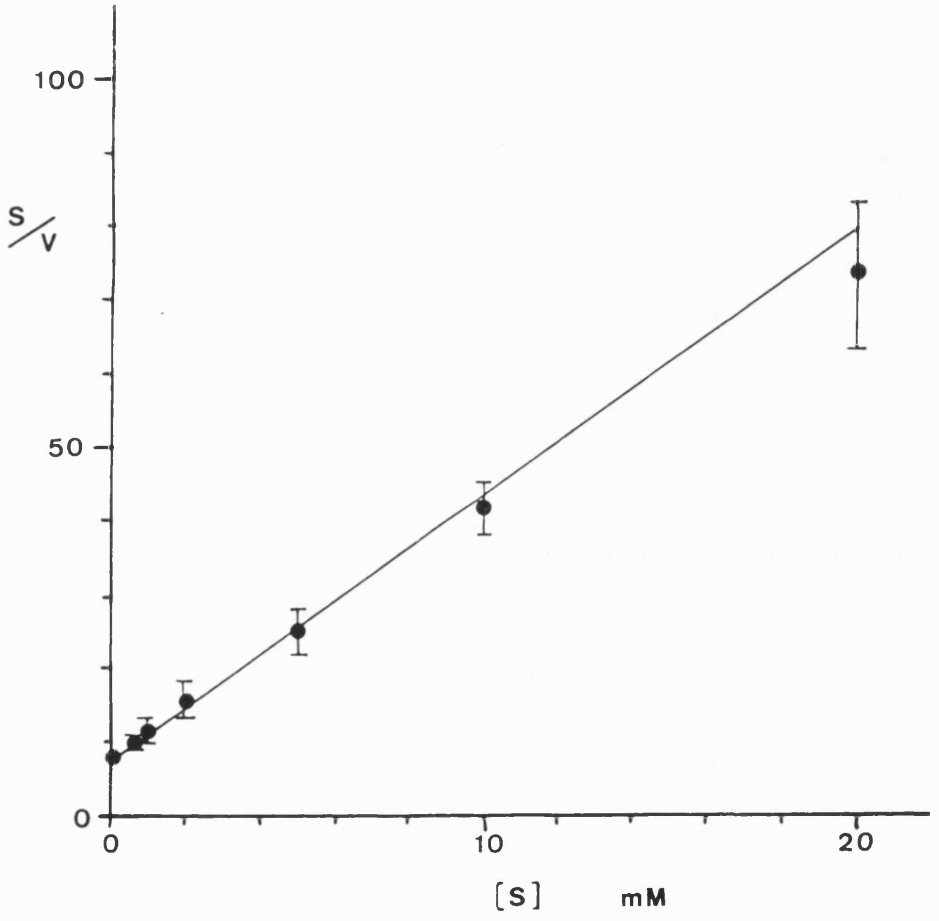


Figure 31: Hanes plot of 6-deoxy-D-glucose uptake under zero-trans conditions using a 4 second single time-point assay at 20°C. K_m = 2.16 ± 0.068mM, V_{max} = 0.279 ± 0.005mM sec^{-1}, n = 6. Line fitted by method of least squares.

The zero-trans uptake parameters were also determined using complete time courses at each concentration of 6-deoxy-D-glucose. It was apparent from the time-courses (Figures 32 - 36 insets) that a 4 second time point would give a significant under-estimate of the initial rate of uptake especially at lower concentrations and calculated initial rates would provide more accurate kinetic parameters for zero-trans uptake.

The calculation of the initial rate used the integrated rate procedure as described by Eilam and Stein (1973). According to this procedure t/C is plotted against $-(\ln (1 - C/S_o) + C/S_o)/C$ where t is the time of uptake, C is the internal substrate concentration and S_o is the external substrate concentration. The integrated rate plots were linear (Figures 32 - 36) and the initial velocity was obtained as the reciprocal of the intercepts on the ordinate axis. Figure 37 shows a plot of S/V vs S for the calculated initial velocities. The zero-trans entry kinetic parameters were: K_m = 1.54 $\pm$ 0.28mM, V_{max} = 0.401 $\pm$ 0.04mM sec^{-1}. These values differed from those obtained using the single time-point assay procedure in that the K_m value was 27% higher and the maximum velocity was 31% lower.

4.3.4: Zero-trans exit experiments

The determination of the kinetic parameters of the inside hexose transporter site was undertaken using the

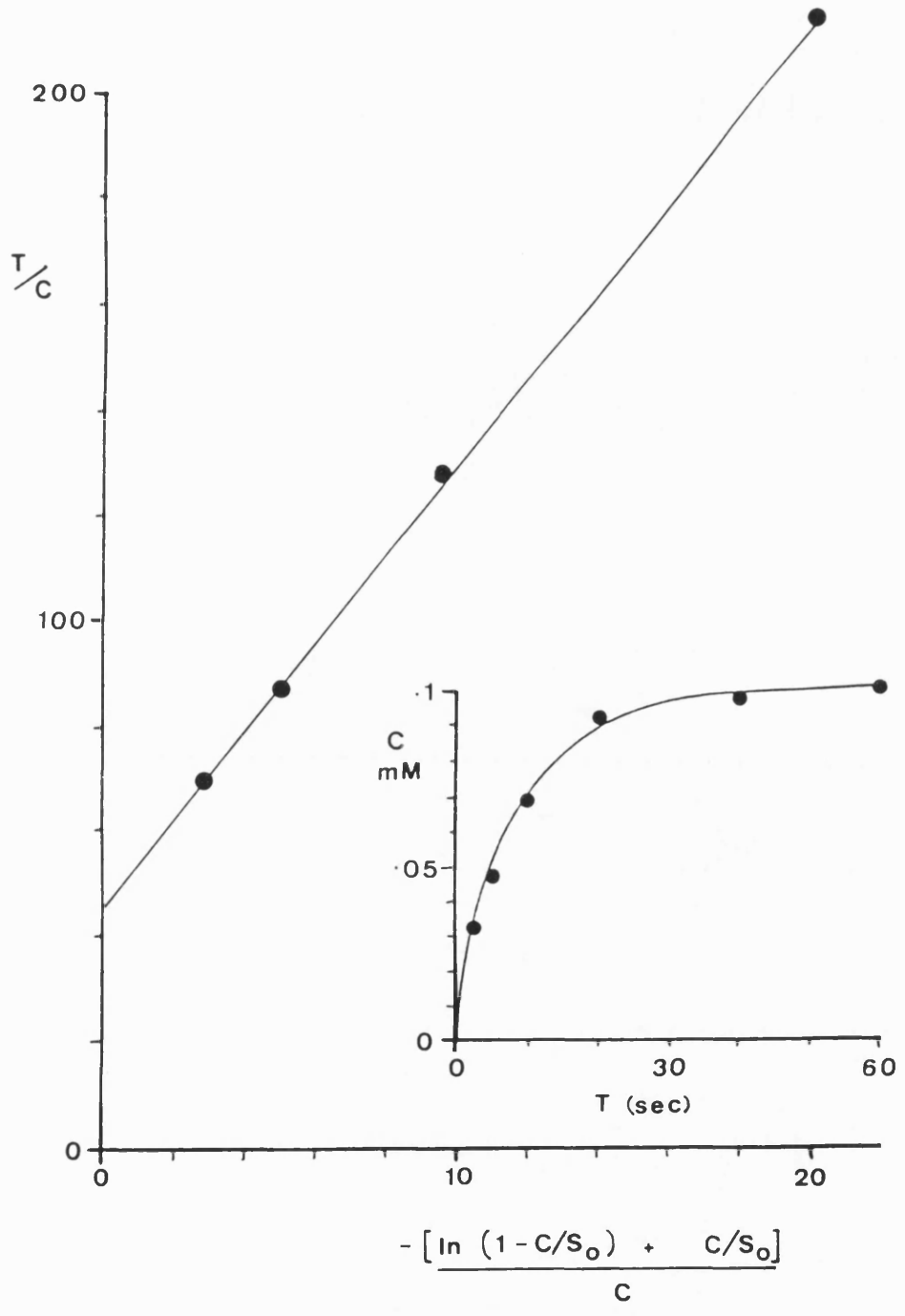


Figure 32: Integrated rate replot of 100μM 6-deoxy-D-glucose. Initial rate (V_o) calculated as 0.024 ± 0.001mM sec^{-1}. Line fitted by method of least squares. Inset: Time-course for 100μM 6-deoxy-D-glucose uptake at 20°C. C is the concentration inside the cells.

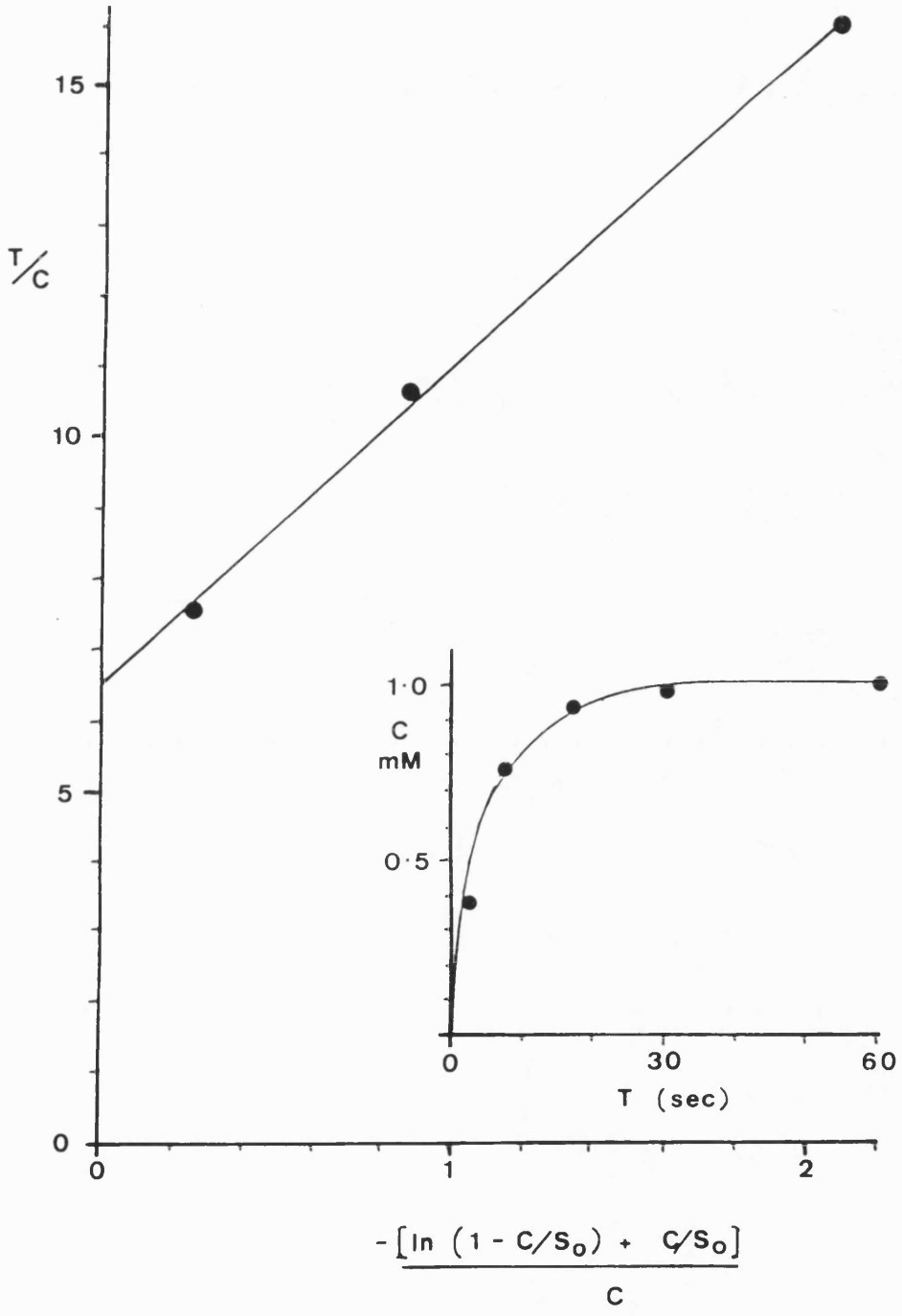


Figure 33: Integrated rate replot of 1mM 6-deoxy-D-glucose. V_o = 0.150 ± 0.003mM sec^{-1}. Line fitted by method of least squares. Inset: Time-course for 1mM 6-deoxy-D-glucose uptake at 20°C. C is the concentration inside the cells.

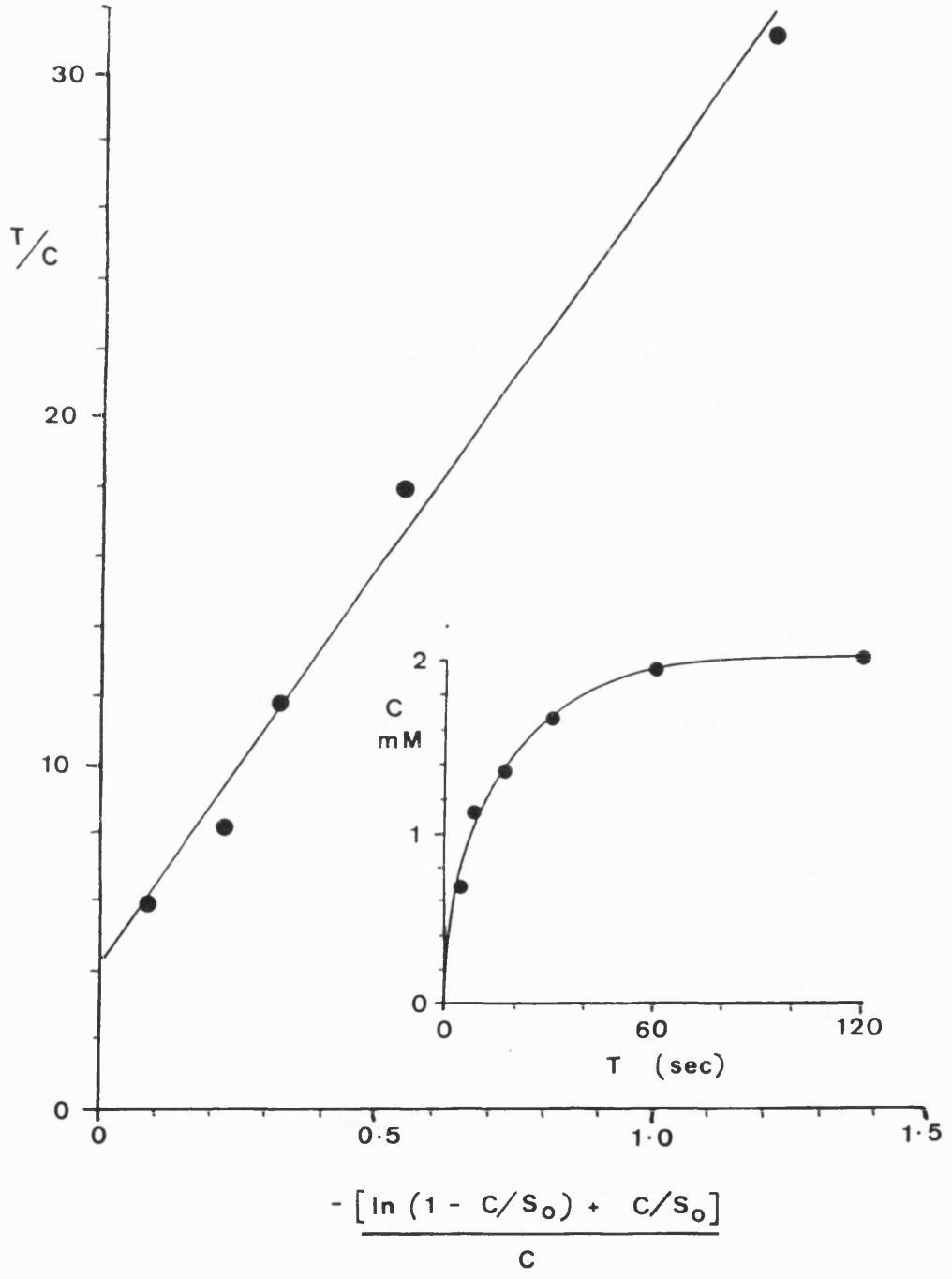


Figure 34: Integrated rate replot of 2mM 6-deoxy-D-glucose. V_o = 0.292 ± 0.013mM sec^{-1}. Line fitted by method of least squares. Inset: Time-course for 2mM 6-deoxy-D-glucose uptake at 20°C. C is the concentration inside the cells.

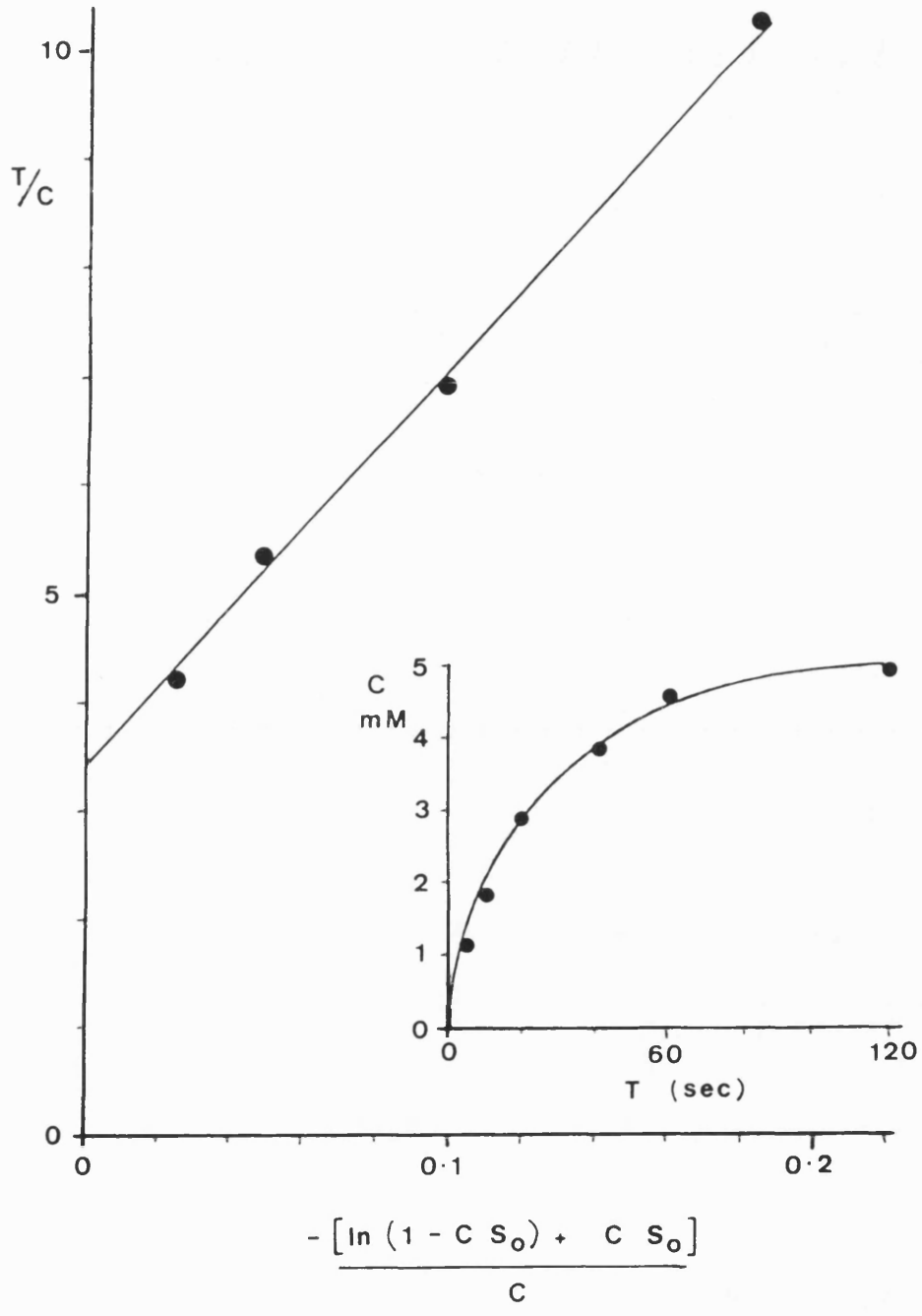


Figure 35: Integrated rate replot of 5mM 6-deoxy-D-glucose. V_o = 0.292 ± 0.013mM sec^{-1}. Line fitted by method of least squares. Inset: Time-course for 5mM 6-deoxy-D-glucose uptake at 20°C. C is the concentration inside the cells.

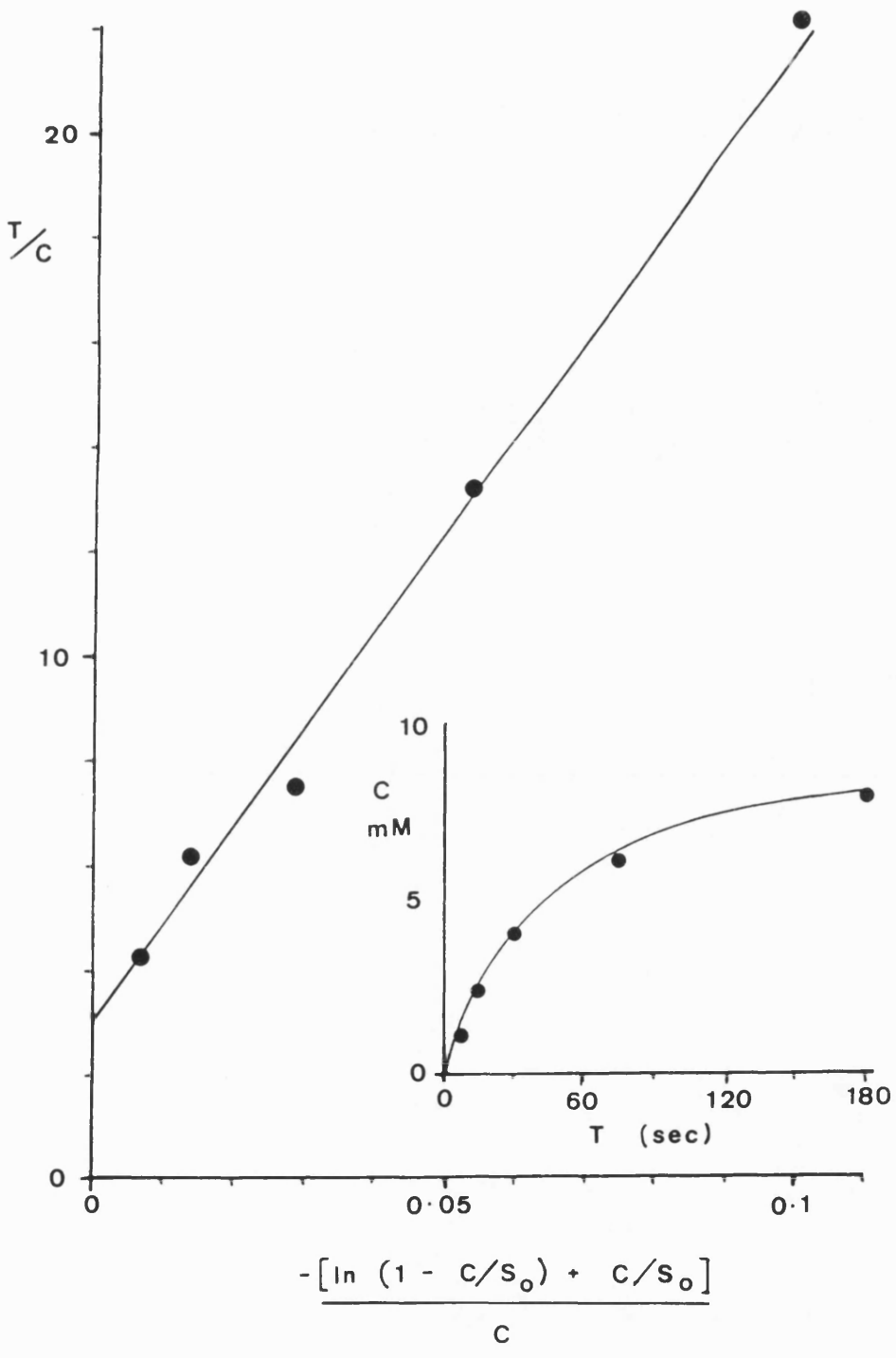


Figure 36: Integrated rate replot of 10mM 6-deoxy-D-glucose. V_o = 0.337 ± 0.047mM sec^{-1}. Line fitted by method of least squares. Inset: Time-course of 10mM 6-deoxy-D-glucose uptake at 20°C. C is the concentration inside the cells.

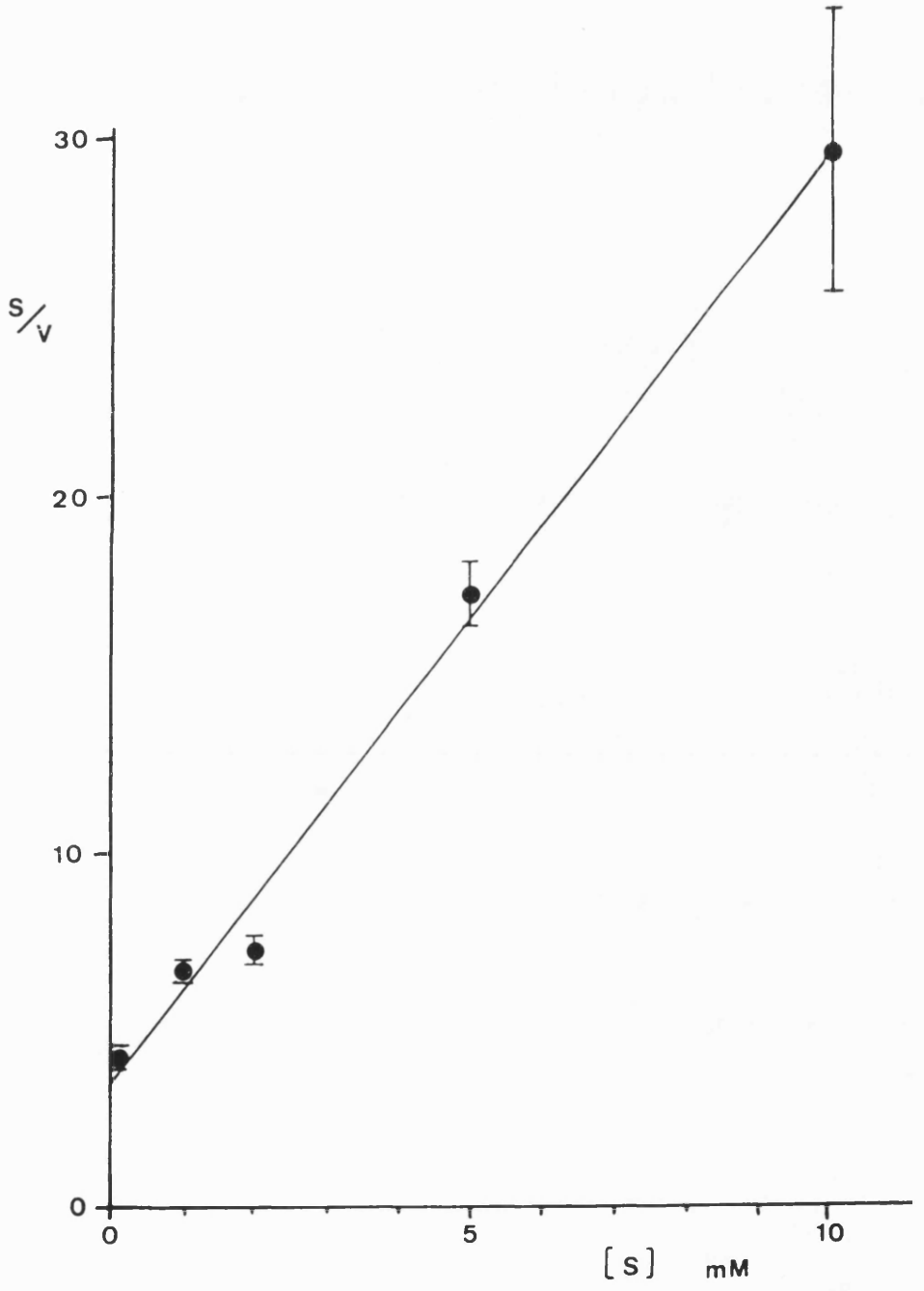


Figure 37: Hanes plot of 6-deoxy-D-glucose uptake under zero-trans conditions at 20°C using the initial rates calculated from the integrated rate replots of uptake time-courses. K_m = 1.54 ± 0.275, V_{max} = 0.401 ± 0.039mM sec^{-1}. Line fitted by method of least squares.

zero-trans exit procedure. A series of efflux time-courses were carried out at different 6-deoxy-D-glucose concentrations and all the data were analysed using the integrated rate procedure of Karlish et al., (1972). The following equation is arranged in the form of a Lineweaver-Burk transformation of the Michaelis-Menten equation:

$$\frac{-\ln \frac{S_t}{S_o}}{S_o - S_t} = \frac{V_{max}}{K_m} \cdot \frac{t}{S_o - S_t} - \frac{1}{K_m}$$

where S_o is the intracellular sugar concentration at time zero, and S_t is the intracellular sugar concentration at time t.

The data shown in Figure 38 represent the efflux time-courses which were replotted using the integrated rate equation as shown in Figure 39. The fitted line was extrapolated to the x and the y axes and the reciprocals calculated to give the following kinetic parameters: K_m = 2.76 ± 0.33mM and V_{max} = 0.279 ± 0.02mM sec^{-1}. These results are similar to the values obtained for the zero-trans entry experiments and suggest that the hexose transport system is symmetrical in its binding site structure.

There were some technical problems with the zero-trans exit procedure due to the low internal volume of the trypanosomes accessible to the analogue. The cells were preloaded with 6-deoxy-D-glucose until equilibrium was reached, followed by a 50-fold dilution in buffer. These

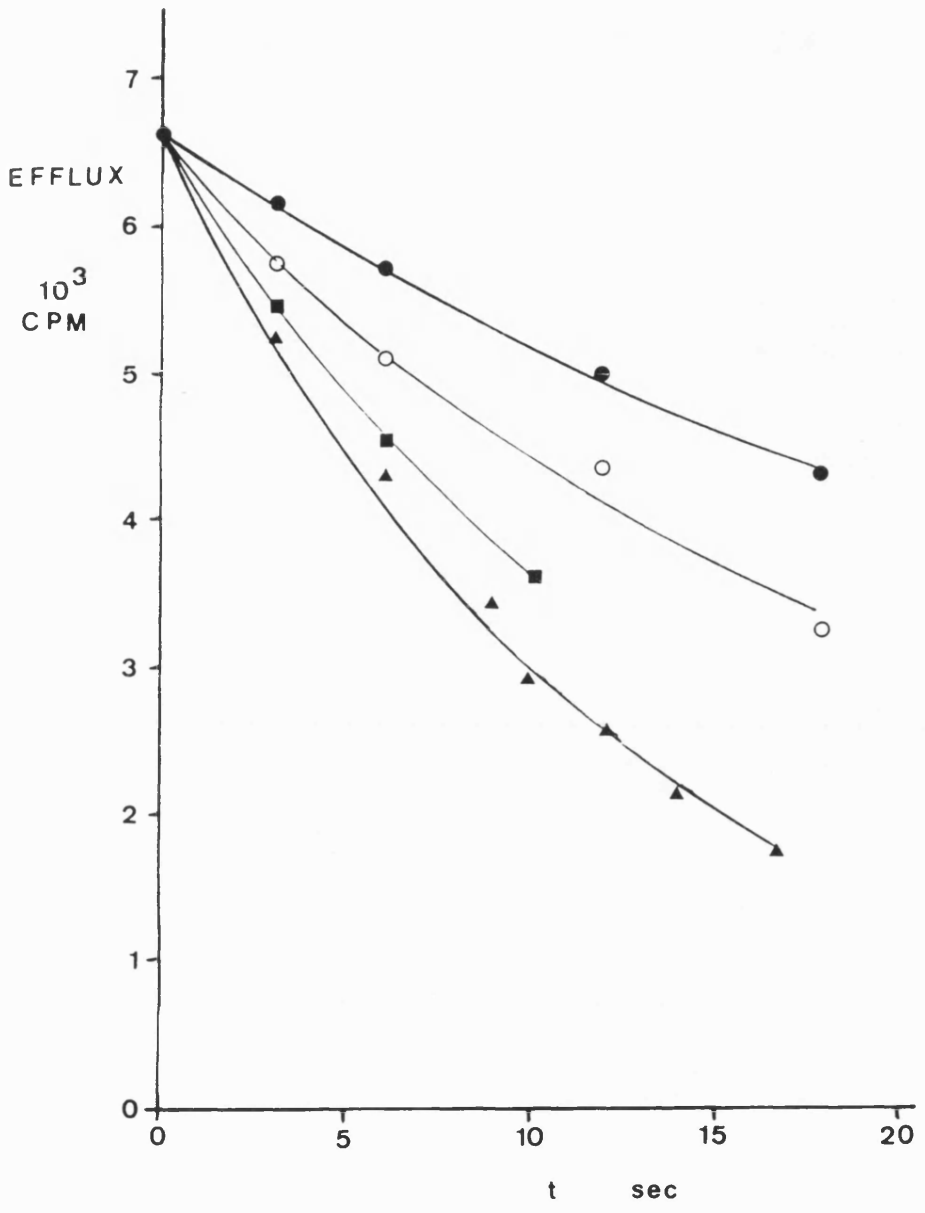


Figure 38: Efflux of 6-deoxy-D-glucose at 20°C from cells preloaded with radio-label for 5 minutes (●) - 10mM, (○) - 5mM, (■) 2mM, (▲) - 1mM 6-deoxy-D-glucose.

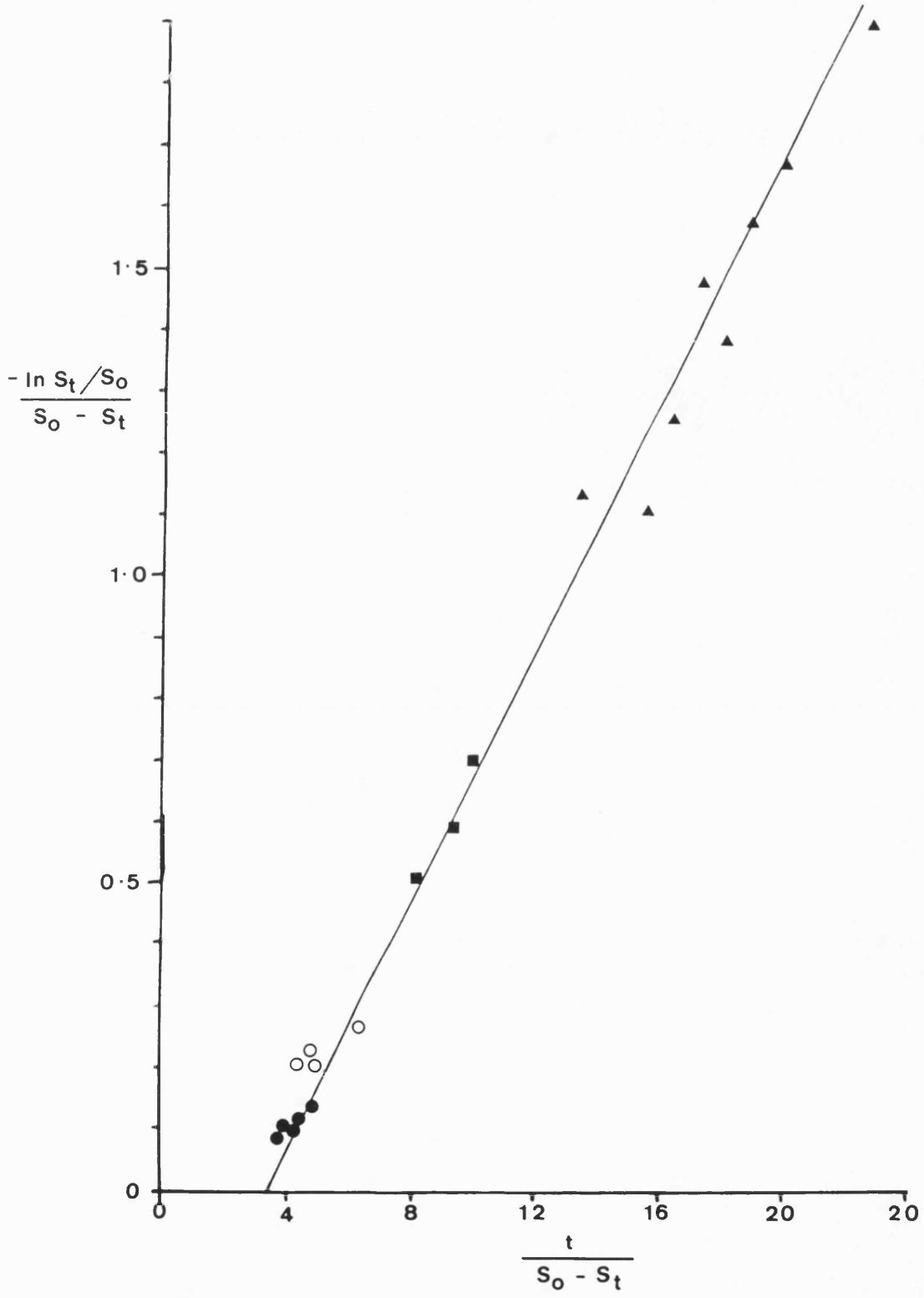


Figure 39: Integrated rate replot of efflux of 6-deoxy-D-glucose at 20°C. (●) - 10mM, (○) - 5mM, (■) - 2mM, (▲) - 1mM. K_m = 2.76 ± 0.33mM, V_{max} = 0.28 ± 0.02mM sec^{-1}. Line fitted by method of least squares.

factors resulted in low CPM values, few cells per pellet and very high cell concentrations when loading with labelled analogue. It was not feasable to increase the initial cell concentration further so great care was taken during the final wash procedures not to inadvertently dislodge and remove the small pellet present.

The possible effect of the difficulties in the procedure should be noted when interpreting the results.

4.3.5: Equilibrium exchange experiments

The kinetics of the hexose transport system were further investigated using the equilibrium exchange procedure. Cells were preloaded with a range of concentrations of unlabelled 6-deoxy-D-glucose followed by the addition of equivalent concentrations of labelled 6-deoxy-D-glucose and the flux of the label was measured.

Initially, a time-course of the uptake of 1mM 6-deoxy-D-glucose under equilibrium exchange conditions was carried out (Figure 40 inset). Under these conditions where no net flux of the analogue occurred, the time-course was found to approximate to a simple exponential curve. This was confirmed by fitting the data to the following equation:

$$\frac{V}{S} = \ln\left[\frac{1}{1-f_i}\right] . \; t^{-1}$$

The resulting replot (Figure 40) produced a straight

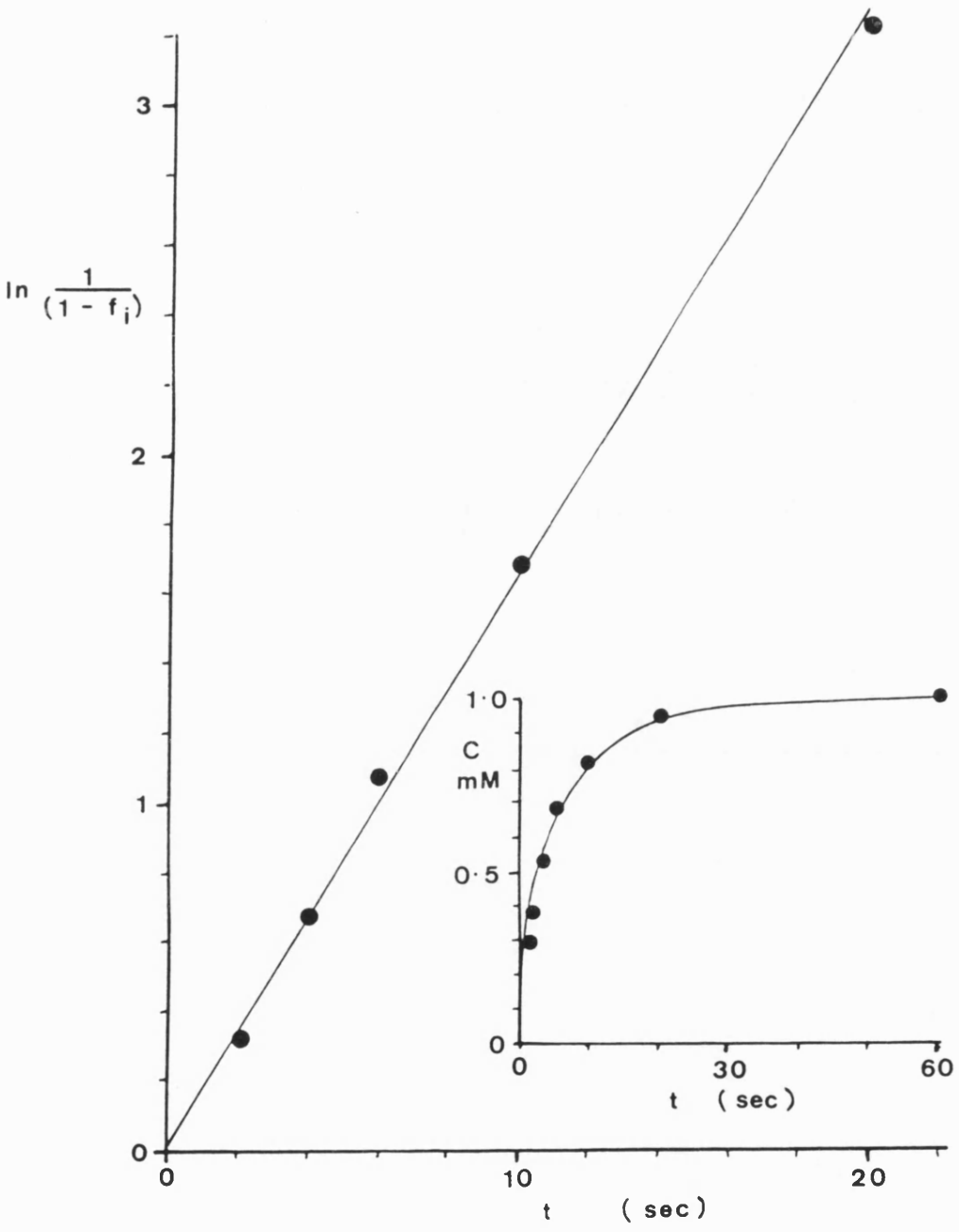


Figure 40: Log plot of 1mM 6-deoxy-D-glucose uptake at 20°C under equilibrium exchange conditions. The figure indicates the exponential nature of the time-course shown in the inset.

line passing through the origin.

Due to the simple exponential nature of the uptake profile, an estimate of the initial rate of uptake at a particular 6-deoxy-D-glucose concentration could accurately be obtained with any single time-point using the relationship:

$$V_o = -\ln (1 - f) \cdot S_o$$

where f is the fractional filling and S_o the outside substrate concentration.

The kinetic parameters for 6-deoxy-D-glucose under equilibrium exchange conditions were obtained using the above relationship and a 4 second single time-point uptake assay. The data was plotted using a Hanes plot (Figure 41) and fitted using the method of least squares.

The kinetic parameters obtained were: K_m = 2.39 $\pm$ 0.41mM and V_{max} = 0.755 $\pm$ 0.084mM sec^{-1}. These values are again similar to those previously obtained reinforcing the evidence for a symmetrical carrier although the maximum velocity was significantly higher than that for zero-trans entry and exit.

Figure 42 shows the time-courses for 1mM 6-deoxy-D-glucose uptake under both equilibrium exchange and zero-trans entry conditions. The result shows that both time-course profiles are virtually superimposable which is also indicative of a symmetrical carrier system.

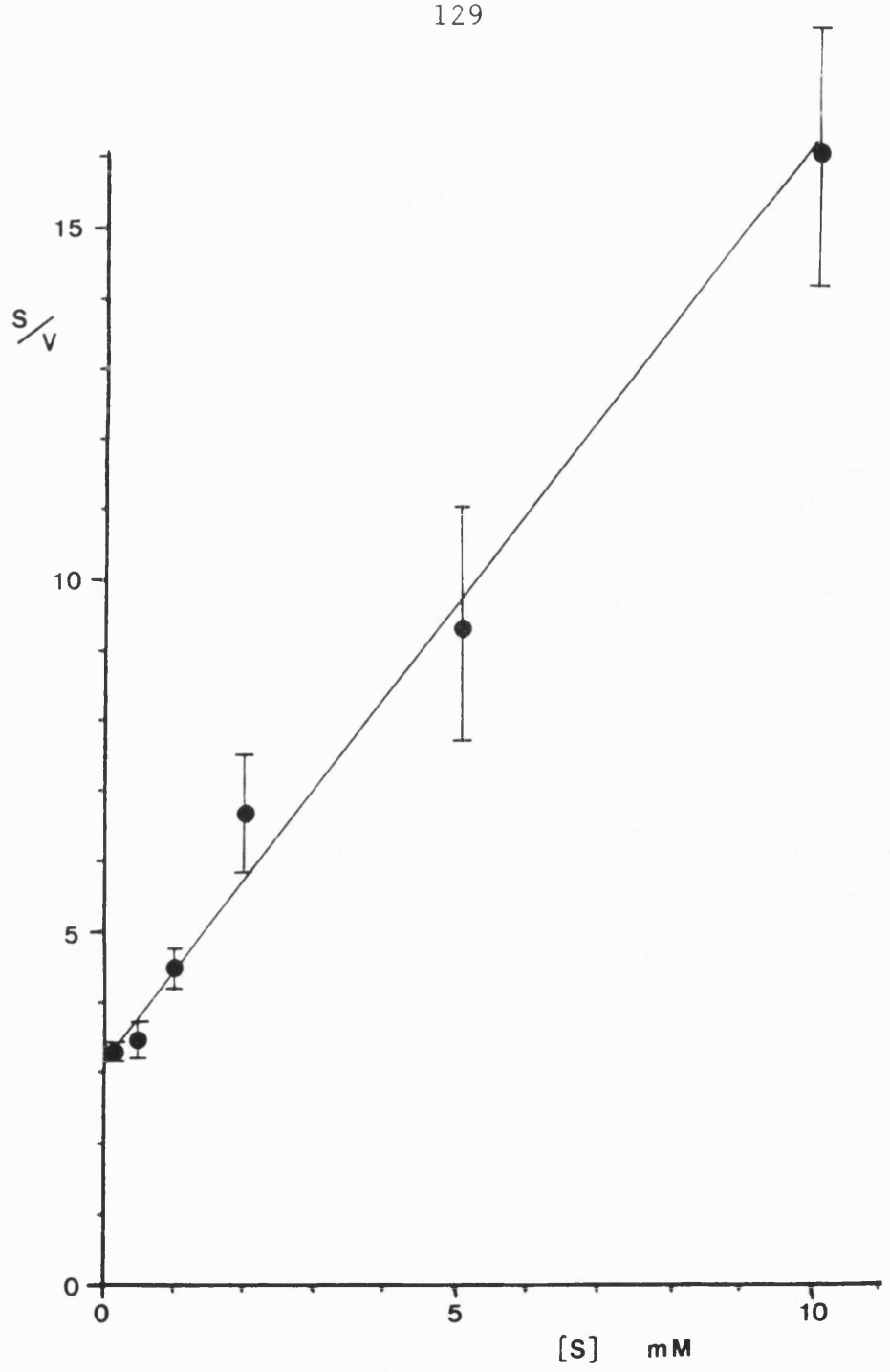


Figure 41: Hanes plot of 6-deoxy-D-glucose uptake vs. concentration at 20°C under equilibrium exchange conditions using a 4 second single time-point. K_m = 2.39 ± 0.41mM, V_{max} = 0.76 ± 0.08mM sec^{-1}, n = 4. Line fitted by method of least squares

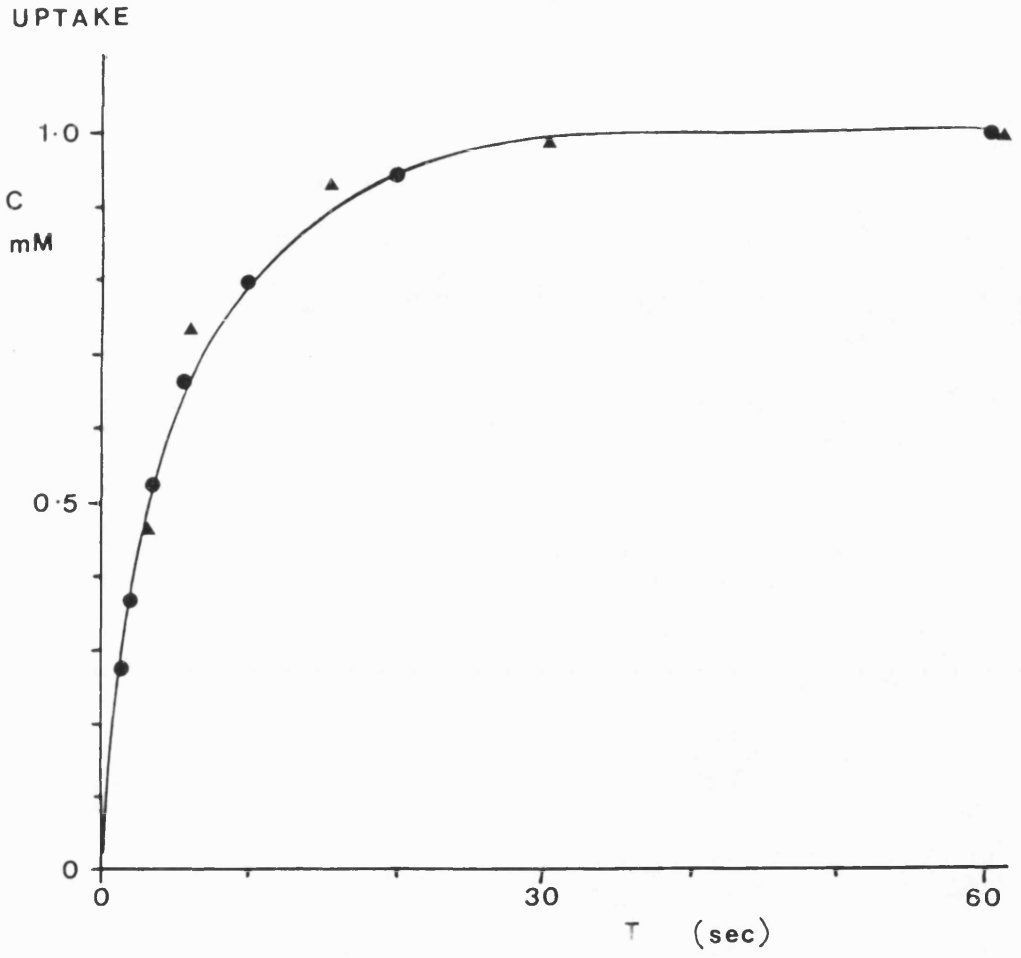


Figure 42: Time-course for uptake of 1mM 6-deoxy-D-glucose under equilibrium exchange conditions (●) and zero-trans conditions (▲) at 20°C.

4.3.6: Infinite cis experiments

A further indication of the K_m for the inside site was obtained using the infinite cis entry procedure. The concentration of substrate used was 40mM 6-deoxy-D-glucose, this being well above the K_m observed for the zero-trans entry experiment. The concentration was thus effectively infinite on the cis side of the transporter.

Figure 43 inset shows a time-course for uptake of 6-deoxy-D-glucose at a 40mM concentration.

The integrated rate equation of Eilam and Stein (1974) was applied to the time-course and the appropriate replot gave a straight line shown in Figure 43.

The intercept on the ordinate axis gave $1/V_{max}$ and the intercept on the abscissa was equal to $-K_m/S_o^2\ (1+S_o/\pi\ )$ where π is the effective osmotic concentration of the medium. The osmotic concentration of the KRP buffer and the substrate was 302 mOsM and the kinetic parameters were: $K_m = 6.21 \pm 1.20$mM and $V_{max} = 0.601 \pm 0.052$mM sec^{-1}. The K_m value is significantly higher than values obtained in the prevous kinetic experiments.

This could be explained by the length of incubation of the cells at the later time-points causing an element of leakage. Also a much lower concentration (50μM) of label was used to determine the equilibrium value for the experiment. Any small error in this value or others

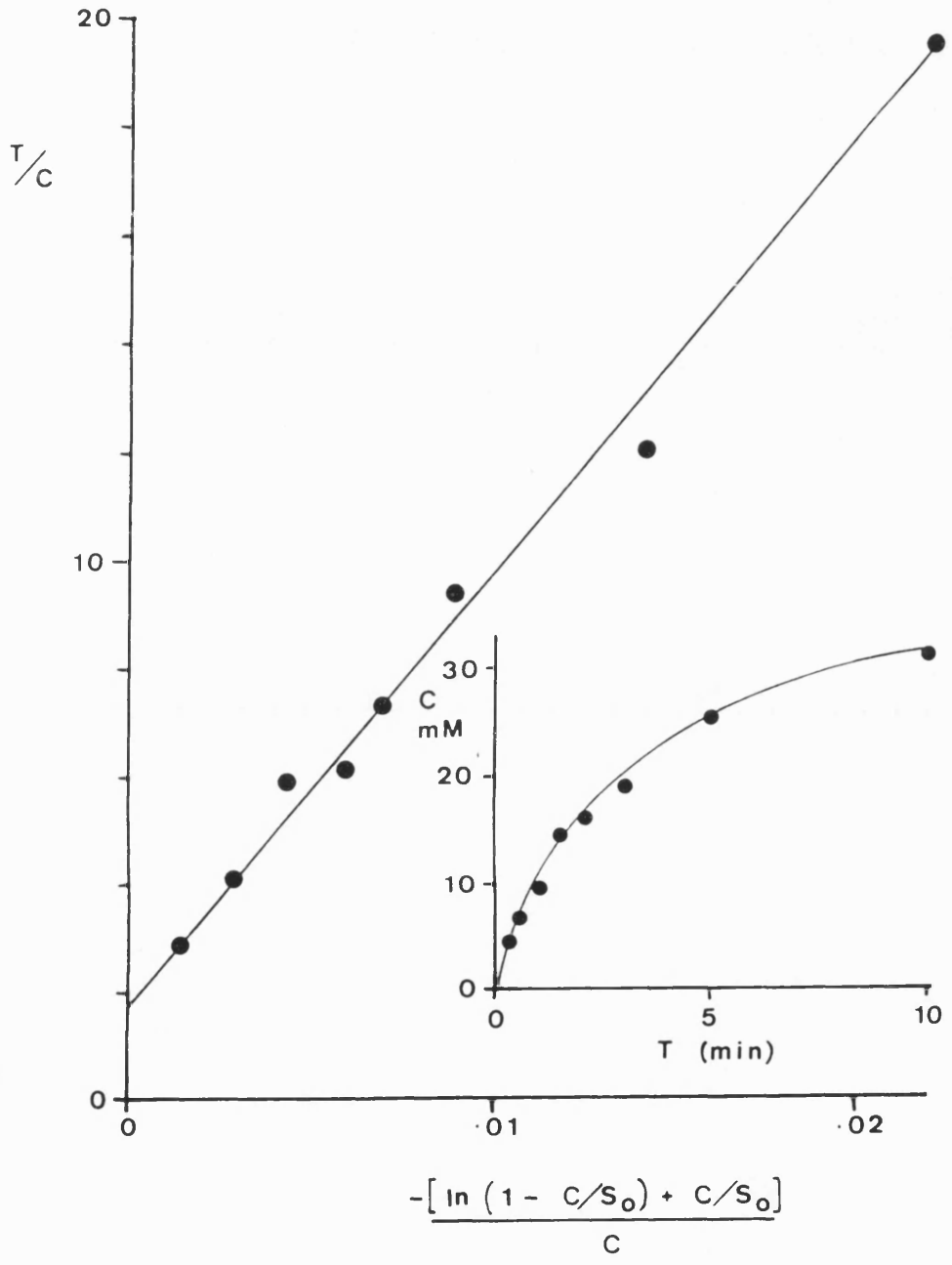


Figure 43: Integrated rate replot of 40mM 6-deoxy-D-glucose. Infinite cis kinetic parameters: K_m = 6.21 ± 1.20mM, V_{max} = 0.601 ± 0.052mM sec^{-1}. Osmolarity = 302 mOsM. Line fitted by method of least squares. Inset: Time-course of 40mM 6-deoxy-D-glucose under infinite cis conditions at 20°C.

KINETIC PROCEDURE	NUMBER OF DETERMINATIONS	AFFINITY CONSTANT K_m mM	MAXIMUM VELOCITY (V_{max}) mM sec^{-1}
Zero-trans entry	6	1.57 ± .28	0.40 ± .04
Zero-trans exit	4	2.76 ± .33	.28 ± .02
Equilibrium exchange entry	4	2.39 ± .41	.76 ± .08
Infinite cis entry	4	6.21 ± 1.20	.601 ± .052

Table 6: Summary of the kinetic parameters of 6-deoxy-D-glucose transport in T. brucei at 20°C.

in the experiment would have a disproportionate effect on the calculation due to the square function in the equation.

4.3.7: Summary of kinetic parameters

The kinetic parameters for K_m and V_{max} using the approaches described previously are summarised on Table 6.

4.4: Specificity of hexose transport in T. brucei

The specificity of hexose transport in T. brucei was analysed using a series of inhibitors of 6-deoxy-D-glucose transport. The aim was to obtain inhibition constants (K_i's) for a range of analogues at each hexose carbon position, investigating both the spatial and hydrogen bonding requirements of the transporter binding site. K_i values were calculated using a range of inhibitor concentrations at a fixed 100μM 6-deoxy-D-glucose concentration. The results were plotted on a Hanes plot and fitted by the method of least squares. When inhibition was less than 10% of the initial rate of 6-deoxy-D-glucose uptake at the highest inhibitor concentration (20-24mM), the K_i value was described as greater than 250mM.

Initial experiments were performed to determine whether preincubation with inhibitor or simultaneous addition of inhibitor would be most appropriate for the assay procedure. The problem with preincubation is that substrates or metabolised substrate analogues would not

reach true equilibrium and the resultant depletion of energy caused by partial metabolism of substrate analogues could affect the rate of transport or the action of the inhibitor.

The effect of preincubation was investigated using 2-deoxy-D-glucose, a substrate analogue known to be rapidly transported and phosphorylated but not further metabolised (Gruenberg et al., 1978). 2-deoxy-D-glucose was preincubated with cells at 20°C for 3 minutes prior to 6-deoxy-D-glucose addition. The K_i value obtained was compared to that produced by simultaneous addition of substrate and inhibitor. The result (Figure 44) indicated that preincubation had little significant effect on the K_i value for 2-deoxy-D-glucose. The small decrease in affinity shown by the inhibitor after preincubation was probably accounted for by the overall drop in inhibitor concentration caused by phosphorylation of transported 2-deoxy-D-glucose.

The above experiment was repeated using 1-deoxy-D-glucose as an inhibitor and surprisingly no significant inhibition occurred after the 3 minute preincubation period even at concentrations up to 200 times the 6-deoxy-D-glucose concentration. Simultaneous addition of inhibitor and substrate did produce a sensible K_i value. Although there was no obvious explanation for the result, it did suggest that simultaneous addition of substrate and inhibitor would be most suitable for the specificity analysis.

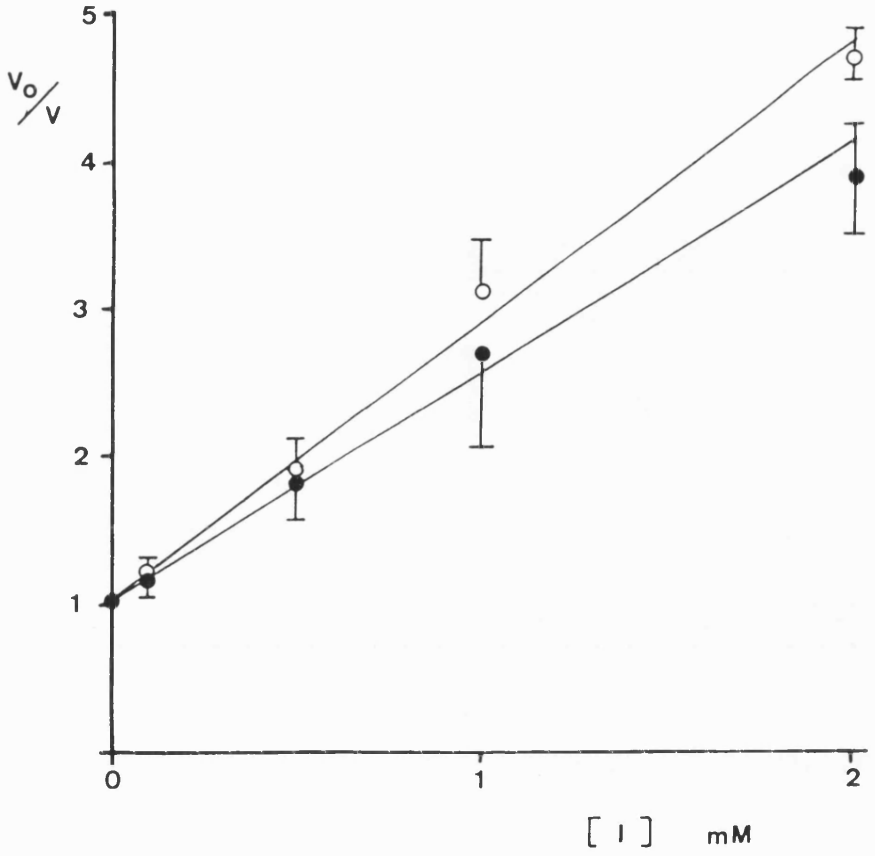


Figure 44: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-glucose without preincubation (○) and with 3 minutes preincubation at 20°C (●), K_i = 0.53 ± 0.08mM, n = 2, (no preincubation) and K_i = 0.63 ± 0.07mM, n = 2, (3 minutes preincubation). Inhibition measured with a 3 second single time-point assay. Lines fitted by method of least squares.

4.4.1: Inhibition of 6-deoxy-D-glucose uptake by substrates of T. brucei metabolism

The natural substrates of T. brucei metabolism, D-glucose, D-fructose, D-mannose and glycerol, were used as inhibitors of 6-deoxy-D-glucose uptake. Glycerol did not significantly inhibit uptake indicating that the hexose transporter has no ability to transport glycerol and its uptake must be via a totally separate transport system.

Figure 45 indicates the K_i values for the other substrates to be: 0.899 ± 0.077mM for D-glucose, 0.670 ± 0.100mM for D-mannose and 2.560 ± 0.004mM for D-fructose. The results indicate that the natural substrates all have a high affinity for the transporter binding site. D-fructose has a significantly lower affinity than D-mannose and D-glucose.

4.4.2: Inhibition by carbon-1 analogues

The carbon-1 analogues used were 1-deoxy-D-glucose, β-fluoro-D-glucose and α-methyl-D-glucoside. Prior to use, β-fluoro-D-glucose was prepared from β-D-glucopyranosyl-fluoro-tetracetate by the addition of 10mM sodium methoxide followed by incubation at room temperature for 2 hours. Previously calculated aliquots of the solution were dispensed into vials and the methanol removed by evaporation. Immediately prior to use, 10µl of buffer was added to the vial to give the correct concentration of β-fluoro-D-glucose for the experiment.

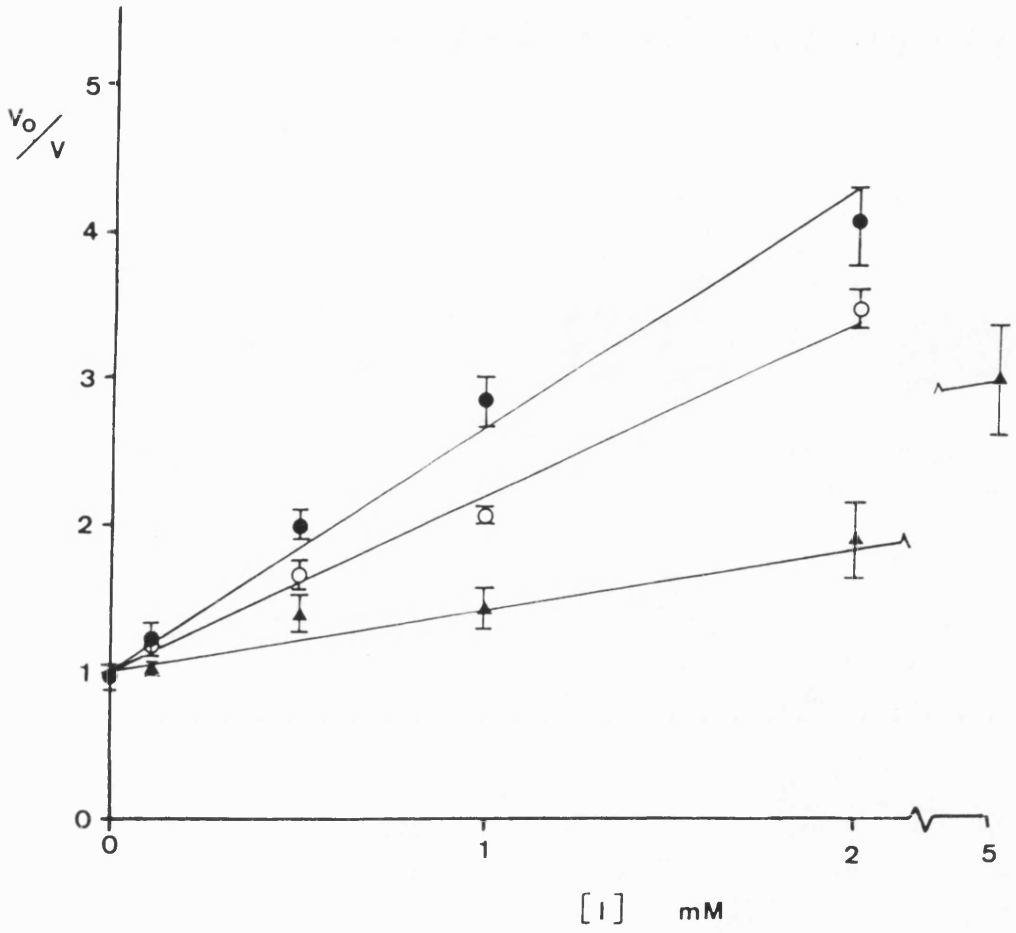


Figure 45: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-mannose (●), K_i = 0.67 ± 0.10mM, n = 2, D-glucose (○), K_i = 0.90 ± 0.04mM, n = 2 and D-fructose (▲), K_i = 2.56 ± 0.40mM, n = 2. Lines fitted by method of least squares.

The pH of the solution was neutral as determined using pH indicator paper.

Figure 46 indicates K_i values of 2.89 ± 0.63mM and 3.65 ± 0.43mM for β-fluoro-D-glucose and 1-deoxy-D-glucose respectively. The K_i value for α-methyl-D-glucoside was found to be greater than 250mM. Similarity of the K_i values for the deoxy- and the deoxy-fluoro-analogue indicate that there is no requirement for a hydrogen bond involving the carbon-1 hydroxyl group. The lack of inhibition by α-methyl-glucoside suggests that there is a close approach to the transport binding site and thus very little room for potential substitution at the carbon-1 position.

4.4.3: Inhibition by carbon-2 analogues

The specificity study at the carbon-2 position used the analogues 2-fluoro-2-deoxy-D-glucose, 2-deoxy-D-glucose, D-glucosamine and N-acetyl-D-glucosamine as inhibitors of 6-deoxy-D-glucose uptake.

The K_i values obtained were 0.44 ± 0.09mM for 2-fluoro-2-deoxy-D-glucose, 0.53 ± 0.08mM for 2-deoxy-D-glucose, 21.34 ± 3.68mM for D-glucosamine and 11.11 ± 1.78mM for N-acetyl-D-glucosamine as shown in Figures 47 and 48.

The K_i values from the deoxy- and the deoxy-fluoro-analogues indicate that no hydrogen bond involving the carbon-2 hydroxyl group occurs. The larger carbon-2 substituted analogues indicate that there is considerable

spatial freedom around the carbon-2 position. The higher affinity shown by N-acetyl-D-glucosamine compared with D-glucosamine may be indicative of a hydrophobic region in the vicinity of carbon-2. Further spatial freedom at the carbon-2 region of the transport site is indicated by the high affinity shown by the substrate D-mannose, the C-2 epimer of D-glucose. However, mannitol, an analogue existing only in the straight chain configuration, did not inhibit 6-deoxy-D-glucose uptake. This suggests that a closed ring structure may be necessary for binding.

4.4.4: Inhibition by carbon-3 analogues

The carbon-3 (C-3) position was analysed using the analogues 3-O-methyl-D-glucose, 3-fluoro-3-deoxy-D-glucose, 3-deoxy-D-glucose and the C-3 epimer of D-glucose, D-allose.

Neither the 3-deoxy-D-glucose nor D-allose significantly inhibited 6-deoxy-D-glucose uptake but 3-fluoro-3-deoxy-D-glucose did inhibit giving a K_i value of 2.31 $\pm$ 0.34mM (Figure 46). The results indicate that the C-3 hydroxyl group is involved in a hydrogen bond necessary for binding to the hexose transporter. The removal of the hydroxyl-group from the correct orientation, ie using 3-deoxy-D-glucose and the C-3 epimer allose as inhibitors, results in a complete loss of affinity. The fluoro-group, which can still participate in hydrogen bonding, only results in a

slight loss of affinity. 3-O-methyl-D-glucose inhibited 6-deoxy-D-glucose uptake giving a K_i of 15.38 ± 0.86mM (See Figure 48). The methyl group indicated a degree of spatial freedom at the C-3 position provided the hydrogen bond can still form. The methyl group did not significantly interfere with the formation of this bond.

4.4.5: Inhibition by carbon-4 analogues

Only limited analysis was performed at the carbon-4 (C-4) position due to the difficulty in synthesising fluoro- and deoxy- analogues at this position. The C-4 epimer D-galactose was found not to inhibit uptake nor did 4,6-ethylidine-D-glucose although this sugar is a C-4/C-6 analogue. Without information from the deoxy- and deoxy-fluoro- analogues, no conclusions as to hydrogen-bonding requirements could be drawn. The lack of inhibition by D-galactose suggests that there is either a very close approach to the binding site at the C-3 position, or a hydrogen bond is necessary, or that both these conditions must be satisfied for binding to occur.

4.4.6: Inhibition by carbon-5 analogues

The sole carbon-5 (C-5) analogue, 5-thio-D-glucose did inhibit uptake with a K_i of 11.67mM as shown in Figure 49. This would indicate that the replacement of the ring oxygen with a sulphur atom does reduce the

affinity of the analogue for the binding site. Hydrogen bonding may occur with the ring oxygen but is not essential for binding to the hexose transporter.

4.4.7: Inhibition by carbon-6-analogues

The effectiveness of 6-deoxy-D-glucose as a substrate for the hexose transporter has already been established. This indicates that a hydrogen bond involving the carbon-6 (C-6) hydroxyl group is not necessary for binding and transport to occur. The analogue 6-fluoro-6-deoxy-D-glucose was not available for study but 6-chloro-6-deoxy-D-glucose had been synthesised and was used as an inhibitor. Figure 47 indicates a K_i value of 0.67 ± 0.09mM. The analogue D-xylose did not however inhibit uptake indicating that the carbon at position 6 is necessary for effective binding to the transporter.

No analogues were available to investigate the spatial characteristics around carbon-6.

4.4.8: Other analogues

The previous sections account for all the analogues tested as inhibitors of 6-deoxy-D-glucose uptake except the disaccharide, maltose. Figure 49 indicates that a K_i of 16.46mM was determined. It is surprising that maltose inhibits 6-deoxy-glucose uptake as maltose, being a disaccharide of D-glucose joined by an α-1,

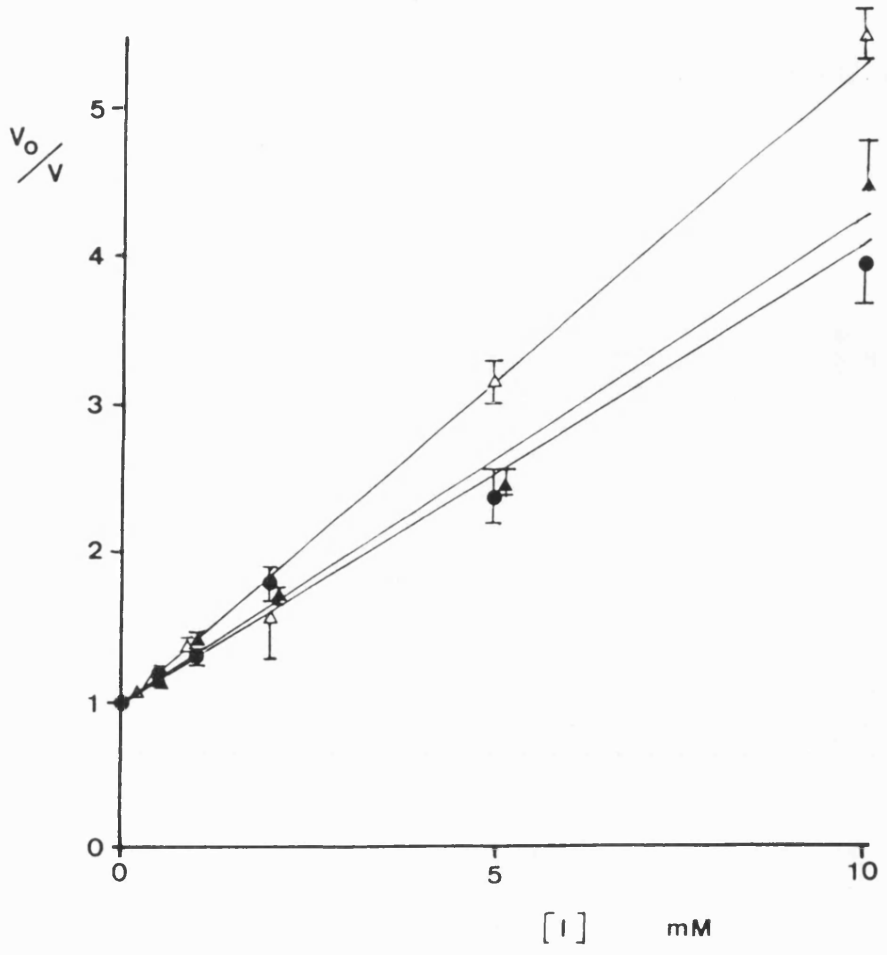


Figure 46: Inhibition of 100μM 6-deoxy-D-glucose uptake by β-fluoro-D-glucose (▲), K_i = 2.89 ± 0.63mM, n = 2, 1-deoxy-D-glucose (●), K_i = 3.65 ± 0.43mM, n = 2 and 3-fluoro-3-deoxy-D-glucose (△), K_i = 2.31 ± 0.34mm, n = 2. Lines fitted by method of least squares.

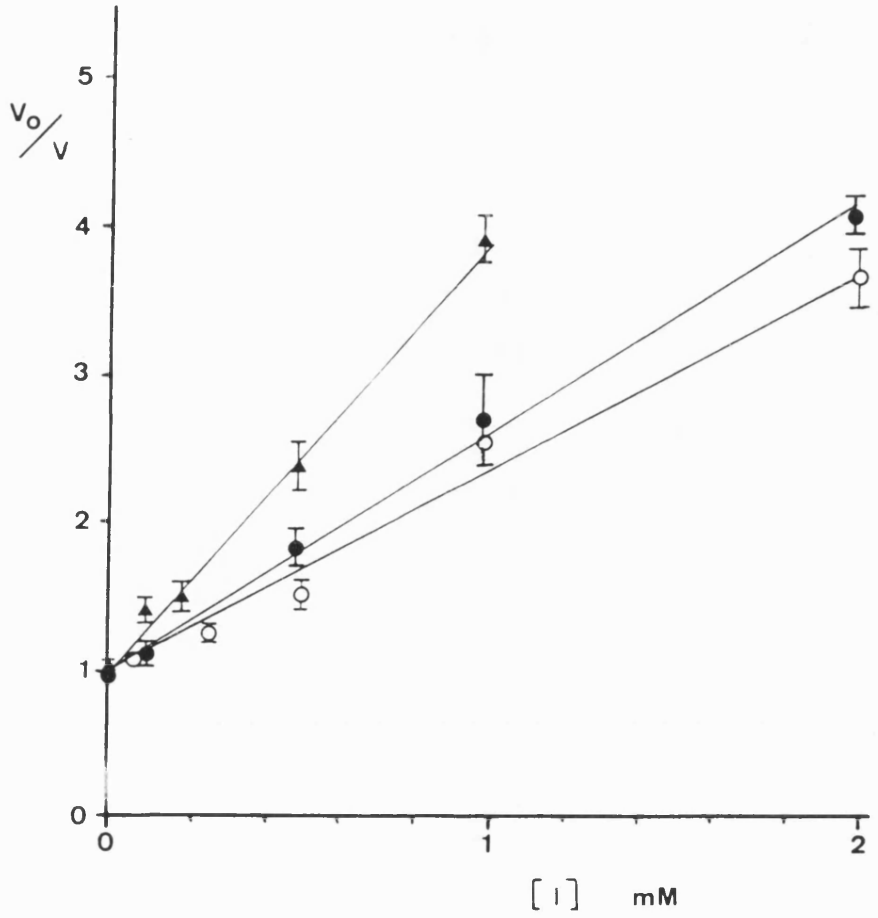


Figure 47: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-glucose (●), K_i = 0.530 ± 0.078mM, n = 2. 2-fluoro-2-deoxy-D-glucose (▲), K_i = 0.444 ± 0.085mM, n = 2. 6-chloro-6-deoxy-D-glucose (○), K_i = 0.670 ± 0.090mM, n = 2. Lines fitted by method of least squares.

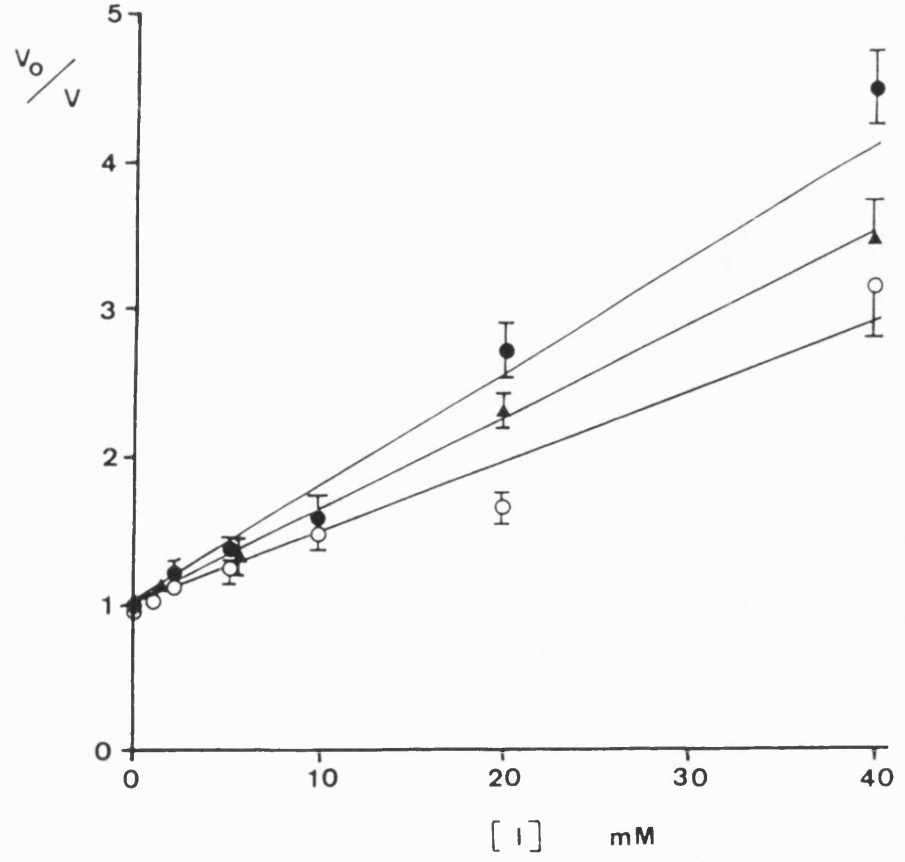


Figure 48: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-glucosamine (o), K_i = 21.34 ± 3.68mM, n = 2. 3-O-methyl-D-glucose (▲), K_i = 15.38 ± 0.86mM, n = 2. N, acetyl-D-glucosamine (●), K_i = 11.11 ± 1.78mM, n = 2. Lines fitted by method of least squares.

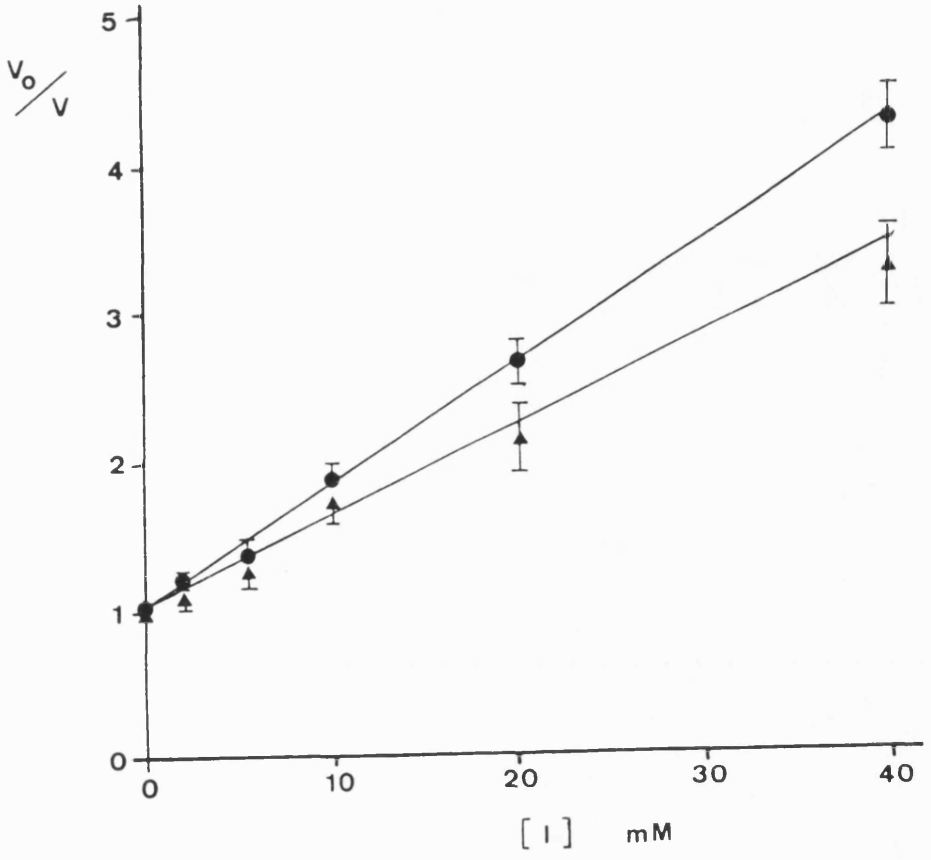


Figure 49: Inhibition of 100μM 6-deoxy-D-glucose uptake by 5-thio-D-glucose (●), K_i = 11.67 ± 0.94mM, n = 2. Maltose (△), K_i = 16.46 ± 1.93mM, n = 2. Lines fitted by method of least squares.

4-glycosidic bond, is either a C-1 or C-4 analogue of D-glucose, both of which are positions demonstrated to have little or no spatial freedom. It is possible that the disaccharide binds in a different fashion, still inhibiting uptake of 6-deoxy-D-glucose.

Another explanation may be the effect of D-glucose impurities in the commercial compound. D-glucose contamination of 1% is usual and, at the concentration range used, D-glucose may have been present in sufficiently high concentrations to account for all the inhibition shown in the plot.

4.4.9: Summary of the specificity study

Table 7 indicates the results of all inhibition studies on the uptake of 6-deoxy-D-glucose and represents the complete specificity analysis as allowed by the availability of analogues for use as inhibitors.

	NAME	INHIBITION CONSTANT mM
Substrates of metabolism	D-glucose	0.90 ± .04
	D-mannose	0.67 ± 0.10
	D-fructose	2.56 ± 0.40
	Glycerol	>250
C1 Analogues	1-deoxy-D-glucose	3.65 ± 0.43
	1-fluoro-1-deoxy-D-glucose	2.89 ± 0.63
	∝-methyl-D-glucoside	>250
C2 Analogues	2-deoxy-D-glucose	0.53 ± .08
	2-fluoro-2-deoxy-D-glucose	0.44 ± .07
	D-glucosamine	21.34 ± 3.68
	N-acetyl-D-glucosamine	11.11 ± 1.78
	Mannitol	>250
C3 Analogues	3-deoxy-D-glucose	>250
	3-fluoro-3-deoxy-D-glucose	2.31 ± 0.24
	D-allose	>250
	3-0-methyl-D-glucose	15.38 ± 0.86
C4 Analogues	D-galactose	>250
	4,6 ethylidine-D-glucose	>250
	Maltose	16.46 ± 1.93
C5 Analogues	5-thio-D-glucose	11.67 ± 0.94
C6 Analogues	6-deoxy-D-glucose	1.54 ± 0.28*
	6-deoxy-6-chloro-D-glucose	0.67 ± 0.09
	D-xylose	>250

* K_m not K_i

Table 7: Inhibition constant (K_i) values for a range of D-glucose analogues determined by inhibition of 6-deoxy-D-glucose uptake using a 3 second uptake assay at 20°C.

CHAPTER 5: DISCUSSION

The transport of sugars into trypanosomes, though clearly an important step in the production of metabolic energy, has received very little previous attention. Gruenberg et al., (1978) stated that the transport process was the rate-limiting step in the glycolytic pathway, the sole source of energy in bloodstream-forms of T. brucei. If this is the case then transport of hexoses is potentially even more important, representing a prime target for the action of trypanocidal agents.

Regulation of the glycolytic flux does not appear to be under enzymic control. Of the classical regulatory enzymes, neither hexokinase, phosphofructokinase (Nwagu and Opperdoes, 1982) nor pyruvate kinase (Flynn and Bowman, 1981), have been found to regulate glycolysis in T. brucei. The hypothesis that the permeation process is rate-limiting has come about almost by default. The conclusions of Gruenberg et al., (1978) were based on indirect evidence of assays which measured steady-state uptake rates rather than initial rates and non-metabolisable substrates were not used. These results indicate that permeation proceeded at a rate slower than metabolism. The transport rate was given as 21nmol/min/ 10^8 cells. It is difficult to see how metabolism of D-glucose can occur at the described rate when permeation proceeds maximally at a rate 4 times more slowly. This suggests that a significant underestimate of the transport flux has been made.

One of the aims of the work in this thesis has been to develop suitable transport protocols which, by

specifically measuring the rate of substrate transport in trypanosomes, can clarify the rate-limiting properties of the translocation step. Initially, studies were centred on the poorly metabolised D-glucose analogue, 1-deoxy-D-glucose (Game et al., 1986). Although useful kinetic parameters for influx were obtained, attempts to characterise efflux and exchange kinetics and carry out inhibition studies were complicated by a significant phosphorylation of the analogue by intact trypanosomes. This is in contrast to the studies using 1-deoxy-D-glucose as a substrate for trypanosomal hexokinase in vitro where no phosphorylation was detected (See Section 4.2.1). This also raised questions on the method of phosphorylation of transported substrates which will be discussed later. Early studies showed that 6-deoxy-D-glucose was an effective inhibitor of 1-deoxy-D-glucose uptake. Since 6-deoxy-D-glucose cannot be metabolised, it was considered an important analogue for the study of transport. As this analogue is not available commercially as a radio-label, the synthesis of tritiated 6-deoxy-D-glucose was regarded as essential to the success of these studies.

5.1: Synthesis of radio-labelled 6-deoxy-D-glucose

Initially, tritiated lithium aluminium hydride ($LiAlH_4$) was used as the means for incorporating radio-activity into the substrate analogue. This involved the synthesis of 6-chloro-6-deoxy-α-methyl-D-glucoside,

protection of free hydroxyl groups and exchange of chlorine with tritium from tritiated $LiAlH_4$ (Section 3.1). Only one attempt at this radio-labelling step was possible due to the withdrawal of commercially available ^3H-$LiAlH_4$. However, on the evidence of this one attempt, any further effort may have been equally unsuccessful. $LiAlH_4$ is inherently unstable and the long shipment time may have accentuated problems with the decomposition of the radio-label. It was apparent from the manufacturers that problems in supply of stable labelled $LiAlH_4$ was responsible for the withdrawal of the product from the market.

The alternative and more expensive radio-labelling procedure involved the exchange of halogen with tritium gas over a palladium catalyst (Amersham procedure Tr-3). The precursor recommended by Amersham for the tritium labelling was 6-iodo-α-methyl-D-glucoside. This was because the iodo- group is the halogen which exchanges most readily with tritium in the presence of the catalyst. The previous approach using a protected chloro-glucoside and ^3H-$LiAlH_4$ for the synthesis of labelled 6-deoxy-D-glucose may, therefore, have been more successful if a protected iodo-glucoside had been synthesised and used rather than a chloro-compound.

The Tr-3 procedure did not require the protection of free hydroxyl groups, however, the investigation in this thesis of suitable methods for protecting hydroxyl groups may be useful for future work involving the

synthesis of C-6 sugar analogues.

Initial attempts to synthesise the 6-iodo derivative used two methods, both of which were unsuccessful. Direct halogenation of sugars using N-iodo succinimide to iodinate α-methyl-D-glucoside in the presence of triphenyl phosphine as described by Hanessian and Lavallee, (1976) was not successful although they described similar reactions which proceeded with high yield. The second method, involving tosylation of the C-6 hydroxyl group followed by replacement with iodine from sodium iodide, failed to yield a single readily purifiable product although there is no reason why the reaction should not be successful if sufficient time were spent optimising the reaction conditions. The availability of previously synthesised 6-Chloro-α-methyl glucoside provided an alternative means for synthesis of the iodo-compound by halogen exchange with sodium iodide. This simple reaction worked well, yielding a single easily-purified product. The subsequent radio-labelling produced a very large quantity of methyl-6-deoxy-D-glucose of high specific activity and, in terms of the radio-chemical yield, was highly successful and cost effective.

5.2: Kinetics of 6-deoxy-D-glucose transport

The transport of 6-deoxy-D-glucose into trypanosomes was found to be extremely rapid at 37°C and it was necessary to reduce the temperature for the kinetic characterisation to 20°C. At this temperature the design

and execution of protocols to measure both uptake and efflux were feasible. From the equilibrium values obtained for 6-deoxy-D-glucose uptake, the water space available to the analogue was calculated as 1.19 ± 0.13µl per 10^8 cells*. This value is comparable to that of Voorheis and Martin, (1980) of 1.75µl per 10^8 cells, but was considerably lower than the value of 9.5µl per 10^8 cells determined by Damper and Patten, (1976). This latter value was used by Gruenberg et al., (1978) to calculate internal concentrations of D-glucose and 2-deoxy-D-glucose after transport had occurred. These calculations will have been considerably affected by the overestimate of the value for the internal cell volume. The internal volume of 5.9µl per 10^8 cells as reported by us (Game et al., 1986) using 1-deoxy-D-glucose was in error due to the trapping of some of the radio-label as sugar phosphate.

The values of the kinetic parameters calculated from both the zero-trans entry and exit experiments were similar. This suggests that a symmetrical carrier is operative in T. brucei. This is confirmed by the results from the equilibrium exchange procedure, as time-courses for uptake under exchange and zero-trans conditions were superimposable using 1mM substrate. Kinetic parameters were also similar although the V_{max} for exchange (0.755 ± 0.084mM sec^{-1}) is significantly higher than that calculated under zero-trans condiitons (0.401 ± 0.04mM sec^{-1}). This suggests that the symmetrical transport system shows slightly accelerated exchange

* n = 6.

although this is only clearly evident at high concentrations. The parameters obtained from the infinite cis entry protocol were also similar to those obtained using the other procedures. The variation seen between the different kinetic parameters is not uncommon in other transport systems (See Tables 8 and 9) and may reflect either the deviation of a particular transport system from the simple carrier model or simply the technical difficulties associated with the experimental protocols themselves.

Other mammalian transport systems have generally been classified as symmetrical or asymmetrical carrier systems. Tables 8 and 9 summarise the kinetics of several mammalian sugar transporters. The human erythrocyte was one of the first and most extensively studied transport systems, but its kinetics are still not understood. The important kinetic features are that asymmetric transport rates are evident. Zero-trans entry is characterised by a low K_m and V_{max} whilst zero-trans exit protocols show a high K_m and V_{max}. However, under infinite cis entry conditions which measure the inside site, a low K_m is apparent (Baker and Naftalin, 1979). These results suggest that a simple carrier model is unable to explain the kinetics of human erythrocyte hexose transport. Asymmetric kinetics have also been shown by human lymphocyte hexose transport (Rees and Gliemann, 1985) although the degree of asymmetry is much less pronounced. The latter experiments were carried out at $37^{o}C$ whilst most erythrocyte experiments were performed

CELL TYPE	HUMAN ERYTHROCYTE		HUMAN LYMPHOCYTE [5]		RAT THYMOCYTE [6]	
SUBSTRATE	D-glucose		3-O-methyl-D-glucose		3-O-methyl-D-glucose	
	K_m (mM)	V_{max} (mM/sec)	K_m (mM)	V_{max} (mM/sec)	K_m (mM)	V_{max} (mM/sec)
Zero-trans entry	1.6 [1]	.60	4.5	.72	7.7	.011
Zero-trans exit	2.5 [2]	2.15	12.9	.22	-	-
Equilibrium exchange	3.4 [3]	6.00	11.4	.69	25.0	.035
Infinite cis entry	2.8 [4]	-	1.5	.65	-	-

Table 8: Kinetic parameters of mammalian hexose transport systems showing asymmetric kinetics. References: 1) Lacko et al., 1972, 2) Karlish et al., 1972, 3) Naftelin and Holman, 1972, 4) Hankin et al., 1972, 5) Rees and Glieman, 1985, 6) Whitesell et al., 1977.

CELL TYPE	RAT ADIPOCYTE [1]				RAT HEPATOCYTE [2]	
SUBSTRATE	3-O-methyl-D-glucose				3-O-methyl-D-glucose	
	BASAL		+ INSULIN			
	K_m (mM)	V_{max} (mM/sec)	K_m (mM)	V_{max} (mM/sec)	K_m	V_{max} (mM/sec)
Zero-trans entry	5.41	0.034	6.10	1.20	20.5	1.37
Zero-trans exit	4.09	0.153	2.66	1.19	21.6	1.53
Equilibrium exchange	4.22	0.058	4.45	0.84	19.0	1.47
Infinite cis entry	9.03	0.006	6.51	0.98	-	-

Table 9: Kinetic parameters of mammalian hexose transport systems showing symmetrical kinetics. Reference: 1) Taylor and Holman, 1981, 2) Craik and Elliot, 1979.

at 25°C. However, it seems likely that kinetic asymmetry shown is also expressed at 37°C although the K_m values for zero-trans exit and equilibrium exchange are lower, (Brahm, 1983). Table 9 lists the kinetic parameters for the rat adipocyte and hepatocyte hexose transport systems. The K_m and V_{max} values shown are similar to the kinetic parameters for T. brucei hexose transport (See Table 6).

The rat adipocyte transports hexose rapidly by facilitated diffusion (Crofford and Renold, 1965a, b). Since D-glucose is rapidly metabolised, studies have been performed using the analogue 3-O-methyl-D-glucose. The rate of transport is markedly increased in the presence of insulin and a number of investigations have suggested that the increase in V_{max} is brought about by the redistribution of transporter between the plasma membrane and an internal storage site (Cushman and Wardzala, 1980). The V_{max} for transport after insulin stimulation is similar to the V_{max} for trypanosomal hexose transport (Table 6). It is clear that on the basis of kinetic studies, hexose transport in T. brucei falls into the same category as adipocytes and hepatocytes in that they display symmetrical transport parameters. The role of rapid transport of hexose into adipocytes is clear, allowing removal of D-glucose from the bloodstream for the storage of energy reserves in the form of fat. This is not true for the erythrocyte which shows little D-glucose metabolism and in which the role of the high capacity transport system remains largely unknown.

In order to compare the kinetic values for 6-deoxy-D-glucose transport in T. brucei with those reported by other workers using different species and sub-species of trypanosomes, it was necessary to recalculate V_{max} values in terms of concentration per second. These calculations used the value for the internal volume of T. brucei determined in this work. The assumption was made that the internal volume of other species of trypanosomes was similar to that of T. brucei. This would be true for T. b. gambiense as it is morphologically indistinguishable from T. brucei but this may not be so for T. equiperdum although a significant difference is unlikely.

The V_{max} of 0.401 $\pm$ 0.04mM sec^{-1} for 6-deoxy-D-glucose calculated from zero-trans uptake procedures at 20°C is significantly higher than the corresponding V_{max} of 0.29mM sec^{-1} calculated by Gruenberg et al. (1978) for D-glucose uptake at 37°C in T. brucei. It is not surprising that the rate of uptake is underestimated under steady-state conditions as backflux may reduce the internal substrate concentration. Also, efflux of radio-labelled pyruvate, the product of D-glucose metabolism under aerobic conditions, may occur. This loss of internal radio-label may occur throughout the incubation period and after the transport process has been 'stopped'. It has been shown (Figure 28) that stopping transport with cold buffer alone is insufficient to prevent efflux of substrate and presumably pyruvate and thus a considerable proportion of transported radio-label will have been lost during the long (10-20 minute) processing times for the cells. The

V_{max} values for substrate uptake in other species of trypanosomes are also lower than reported in this work. Sanchez and Read, (1969) reported a V_{max} equivalent to 0.05mM sec^{-1} at 37°C in T. equiperdum and Southworth and Read, (1969) reported a V_{max} of 0.33mM sec^{-1} at 37°C in T. gambiense. The methods for measurement of uptake used were similar to those used by Gruenberg et al., (1978).

The kinetic parameters for D-glucose metabolism at 20°C as measured in the oxygen electrode were K_m = 0.387 ± 0.11mM and V_{max} = 20.3nmoles/min/10^8 cells. Since the cell volume is 1.2µl per 10^8 cells, the V_{max} for D-glucose metabolism is 0.282mM sec^{-1}. Thus the K_m is only slightly lower than the K_i for D-glucose inhibition of 6-deoxy-D-glucose transport and the V_{max} is only slightly lower than the V_{max} for 6-deoxy-D-glucose transport. As the physiological concentration of D-glucose in the blood is approximately 5mM (Whitfield, 1979), transport will occur at close to maximum velocity as the concentration of D-glucose in the blood is well above the K_i for D-glucose inhibition of 6-deoxy-D-glucose. Thus, allowing for some backflux of D-glucose transport which may reduce the available D-glucose for metabolism, it seems likely that transport of sugars into T. brucei is rate-limiting for metabolism. This comparison is based on measurements made at 20°C but is also likely to apply at 37°C. The idea that transport is rate-limiting is supported by the fact that the metabolism of D-fructose is slower than D-mannose and D-glucose (Seed et al., 1965).

Inhibition studies on 6-deoxy-D-glucose uptake have shown a higher K_i value for D-fructose compared with the other sugar substrates (Table 7).

The rate-limiting nature of sugar uptake in T. brucei has important implications on the usefulness of the translocation step as a site for anti-trypanocidal agents. Despite transport being rate-limiting for metabolism, it is not a slow step and this is consistent with the high metabolic rate of trypanosomes compared with mammalian tissues. There are presumably a large number of D-glucose transporters and T. brucei may represent a rich source of this type of hexose transporter for more detailed study.

5.3: Specificity of hexose transport in T. brucei

The specificity of hexose transport was analysed using a series of inhibitors of 6-deoxy-D-glucose transport. The hydrogen bonding requirements were investigated using deoxy and deoxy-fluoro analogues of D-glucose. The fluorine atom is capable of forming a hydrogen bond with a neighbouring hydrogen atom but not a neighbouring oxygen atom. Where possible, spatial constraints on the hexose binding site were investigated using analogues with bulky groups substituted at the appropriate positions. Inhibition of 6-deoxy-D-glucose uptake was expressed by measurement of half-maximal inhibition constants (K_i's) (See Table 7).

There are only four substrates of metabolism in

T. brucei: D-glucose, D-mannose, D-fructose and glycerol. Of these, glycerol did not significantly inhibit uptake of 6-deoxy-D-glucose. This indicates that the hexose transporter has no ability to transport glycerol. Other workers have found that glycerol inhibits uptake of sugars into trypanosomes. Ruff and Reed, (1974) suggested the presence of a separate glycerol transporter but indicated that glycerol could also enter the cell via a sugar transport site in T. equiperdum. Similar findings were shown in T. b. gambiense (Southworth and Read, 1969; 1970). The procedures for the measurement of uptake used by these workers were such that only phosphorylated metabolites would be retained within the cell after the long cell processing times used in the experiments. The inhibitory effect of glycerol is thus most likely to have been at the metabolic level. Under anaerobic conditions, high levels of glycerol are toxic to trypanosomes. Glycerol transported into the cells would rapidly deplete ATP levels via the action of glycerol kinase (Opperdoes and Borst, 1977). The loss of ATP would result in the reduction of other phosphorylated substrates and hence show an apparent inhibition of substrate uptake. The assay procedures used by previous workers would result in anaerobic conditions for at least part of the incubation period. For example, the inhibition experiments used 2ml of incubation medium containing 1mM substrate and at least 6×10^8 trypanosomes. Assuming metabolism of 1mM D-glucose occurs at half the

maximum rate calculated by Flynn and Bowman (1973), approximately 540nmoles of substrate are metabolised during the 2 minute incubation period. Since 1 mole of molecular oxygen is consumed per mole of D-glucose metabolised, this would result in the consumption of 540nmoles of O_2. Buffer saturated with air at 37°C contains oxygen at a concentration of 240µM (Robinson and Cooper, 1970) which, in 2ml of medium, is 480nmoles of oxygen. Thus all oxygen would be used before the termination of incubation and anaerobic conditions would occur. The concentration of oxygen in the buffer is also likely to be significantly less than 240µM at the start of incubation because 1ml of medium containing the cells was preincubated to remove D-glucose from the intracellular and extracellular space prior to the addition of the cells to buffer containing the substrate and inhibitor.

Later work by Gruenberg et al., (1976) showed that glycerol inhibition of D-glucose uptake displayed different kinetics to glycerol inhibition of 2-deoxy-D-glucose. This is indicative of the problems of measuring transport rates using substrates which are metabolised. Gruenberg et al., (1976) concluded that glycerol inhibition was occurring at the metabolic level rather than at the carrier site and went on to propose a separate asymmetric glycerol permeation process (Gruenberg et al., 1980).

In the present study, the use of non-metabolised

6-deoxy-D-glucose as a substrate for hexose transport has clearly shown that glycerol does not interact with the hexose transporter.

5.3.1: Specificity at carbon-1 (C-1)

The results obtained using the fluoro and the deoxy-fluoro analogues indicate that no hydrogen bond is directed towards the oxygen of the C-1 hydroxyl group. However, the affinity of 1-deoxy-D-glucose is low compared with that of D-glucose which suggests that a hydrogen bond may be formed between the hydrogen of the C-1 hydroxyl group and an electronegative group on the binding site. A close approach to the binding site is indicated at the C-1 position due to the lack of inhibition by α-methyl-D-glucoside and thus no space would appear to be available for the accomodation of bulky substituents. In the human erythrocyte, a hydrogen bond is directed to the C-1 hydroxyl group but the close approach to the transporter is also indicated (Barnett et al., 1973a). Similar specificity requirements have also been shown for the rat adipocyte (Holman et al., 1981) and the intestinal basolateral membrane hexose transport system (Wright et al., 1980). The sodium-dependent active sugar cotransporter found in the brush border of intestine and kidney tubules (Crane, 1960) differs in its specificity with both α- and β-methyl glucosides strongly inhibiting sugar uptake. The glycoside phloridzin, a potent inhibitor

of active sugar cotransport (Alverado and Crane, 1962) can also be considered a C-1 analogue with a phloretin moiety attached to the carbon-1 of D-glucose. This must reflect the spatial freedom around the C-1 position in the sodium-dependent cotransport system. The inhibitory effect of phloridzin in the trypanosomal system where such spatial freedom is not evident may be due to the interaction of the phloretin component of the glycoside. Phloretin itself could not be tested as an inhibitor at the concentrations used with phloridzin due to its low aqueous solubility.

The trypanosomal transporter appears to accept both pyranose ring structures and certain open chain structures as 1-deoxy- and 1-fluoro-1-deoxy-D-glucose are fixed in the ring form. D-fructose can form an open chain structure resembling the pyranose ring conformation and is accepted quite well by the transporter. It may be that this open chain structure is responsible for the higher K_i value and slower metabolism compared with D-glucose and D-mannose. In the mammalian transport systems D-fructose generally has a poor affinity for the hexose transporter. (Crane, 1960; Wright et al., 1980).

5.3.2: Specificity at carbon-2 (C-2)

The inhibition results of the carbon-2 epimer. D-mannose, the deoxy and the deoxy-fluoro analogues indicate that no hydrogen bonding occurs at the C-2

position. This is also the case for the erythrocyte and adipocyte transporters (Barnett et al., 1973a; Holman et al., 1981) but is markedly different from the intestinal sodium cotransport system (Crane, 1960) where an absolute requirement for the gluco-configuration at C-2 is shown. The trypanosomal transporter has only moderate affinity for D-glucosamine (K_i = 21.34mM) which may indicate poor acceptance of the protonated and charged amino group. This affinity is partially restored for N-acetyl-D-glucosamine (K_i = 11.11mM). It may be that considerable spatial freedom is available at the C-2 position in contrast to the mammalian transproters. In the human erythrocyte transporter, 2-O-methyl-D-glucose has 20-fold less affinity for the transporter than D-glucose (Barnett et al., 1973a). The C-2 position may represent a site for selective binding of bulky substituents to the trypanosomal transporter.

5.3.3: Specificity at carbon-3 (C-3)

The inhibiton constants of 3-deoxy-D-glucose and 3-deoxy-3-fluoro-D-glucose (no inhibition and 2.31 $\pm$ 0.34mM respectively) indicate a requirement for a hydrogen bond at the C-3 position as the fluoro atom restores the loss of affinity shown by the deoxy-analogue. However, the affinity of the fluoro- analogue is between 2 and 3-fold lower than that of D-glucose. This could indicate additional hydrogen bonding to the hydrogen atom of the C-3 hydroxyl group. 3-O-methyl-D-glucose, which retains the oxygen of the hydroxyl group

has only moderate affinity for the transport site (K_i = 15.38mM). This is probably a consequence of spatial interference by the methyl group but may also be due to the absence of the hydrogen of the C-3 hydroxyl group. 3-O-methyl-D-glucose is rapidly transported by erythrocytes and adipocytes and is generally described as non-metabolised (Whitesell and Gliemann, 1979; Gliemann et al., 1972). We have indirect evidence that 3-O-methyl-D-glucose is phosphorylated by T. brucei. The results shown in Figure 20 indicate that 3-O-methyl-D-glucose behaves exactly as 2-deoxy-D-glucose, an analogue known to be phosphorylated, when uptake is measured following, or without a 3 minute preincubation period. Under the conditions used, only phosphorylated substrate was retained within the cells and this trapped radio-label was reduced to a leakage level when preincubation depleted ATP levels and no phosphorylation could occur.

5.3.4: Specificity at carbon-4 (C-4)

Deoxy- and deoxy-fluoro analogues were not available at the C-4 position. However, the total lack of inhibition or transport by D-galactose suggests that a gluco-conformation may be essential for interaction with the transporter. The lack of inhibition by 4,6-O-ethylidene-D-glucose was probably due to steric interference by the ethylidene group suggesting a close approach to the transporter at C-4. Considerable

spatial freedom and limited hydrogen bonding is seen at the C-4 position in mammalian transport systems.

5.3.5: Specificity at carbon-5 (C-5)

The analogue 5-thio-D-glucose showed moderate inhibition (K_i = 11.67mM) which indicates that the ring oxygen is not an essential requirement for binding. However, a ring oxygen may be involved in a hydrogen bond since the 5-thio derivative has 10-fold lower affinity than D-glucose.

5.3.6: Specificity at carbon-6 (C-6)

The C-6 hydroxyl group is not required for hydrogen bonding since the K_m for 6-deoxy-D-glucose has only 1.5-fold less affinity than D-glucose. Some hydrophobic bonding is likely at this position since D-xylose, on which the C-6 hydroxy-methyl group is absent, shows no affinity for the transporter. Also, 6-chloro-6-deoxy-D-glucose has a lower affinity than D-glucose.

In the human erythrocyte, increasing the bulk of alkyl groups attached at C-6 resulted in increased affinity for the binding site (Barnett et al., 1973a). This was thought to be due to an interaction with a hydrophobic region adjacent to the C-6 position. A similar situation exists in the adipocyte where 6-O-pentyl-D-galactose has higher affinity than D-galactose, but 6-O-methyl-D-galactose showed lower affinity

than both D-galactose and the 6-O-pentyl derivative (Holman et al., 1981).

It is clear from these results that the specificity of the T. brucei hexose transporter differs in many respects from mammalian transport proteins. Models for the binding of D-glucose to the carrier have been proposed on the basis of specificity results and Figure 50 shows a model of the glucose-carrier complex for the human erythrocyte. A similar model has also been proposed for the rat adipocyte (Holman et al., 1981). The model shows that carbon-4 and to some extent carbon-6 are outward-facing and thus able to accept bulky alkyl groups without loss of affinity for the binding site.

A possible model for the trypanosomal sugar-carrier complex is shown in Figure 51. A major difference between the mammalian and trypanosomal transport models is that the C-4 position does not appear to be outward-facing in the trypanosome. Evidence suggests a close approach of carbon-4 to the binding site but more information would be necessary to prove this conclusively. It is possible that the carbon-2 position is outward-facing in the trypanosomal binding site which would indicate a reversal of the sugar orientation in the site. Again more information is needed on the extent of the spatial freedom at the C-2 position which thus far represents the only site where the specificity requirements are less rigorous than in the mammalian

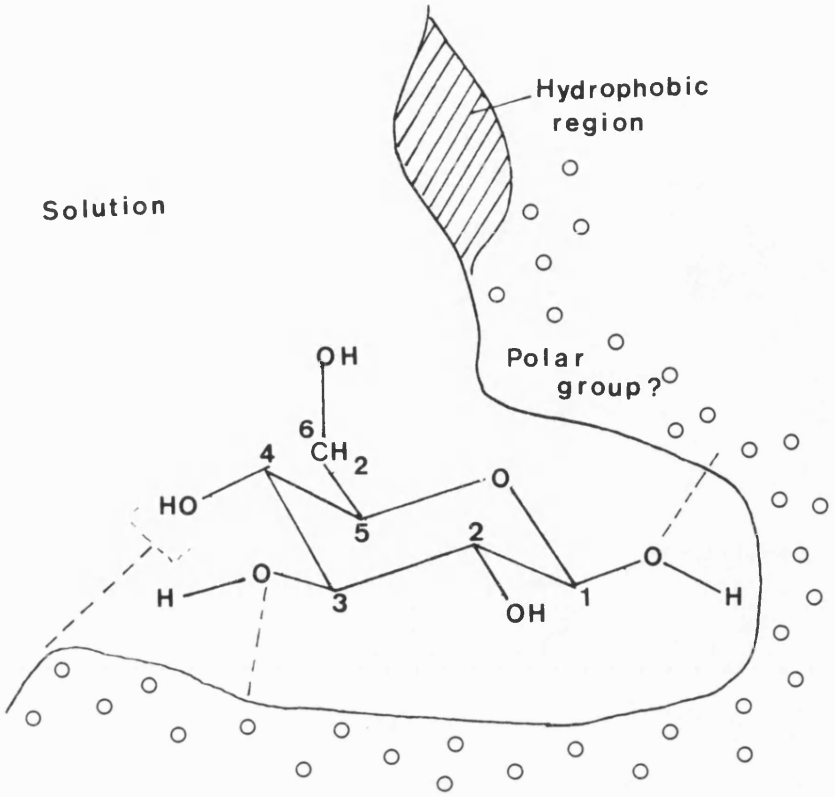


Figure 50: Model of the D-glucose-carrier complex in the human erythrocyte (from Barnett et al., 1973). Broken lines indicate hydrogen bonds.

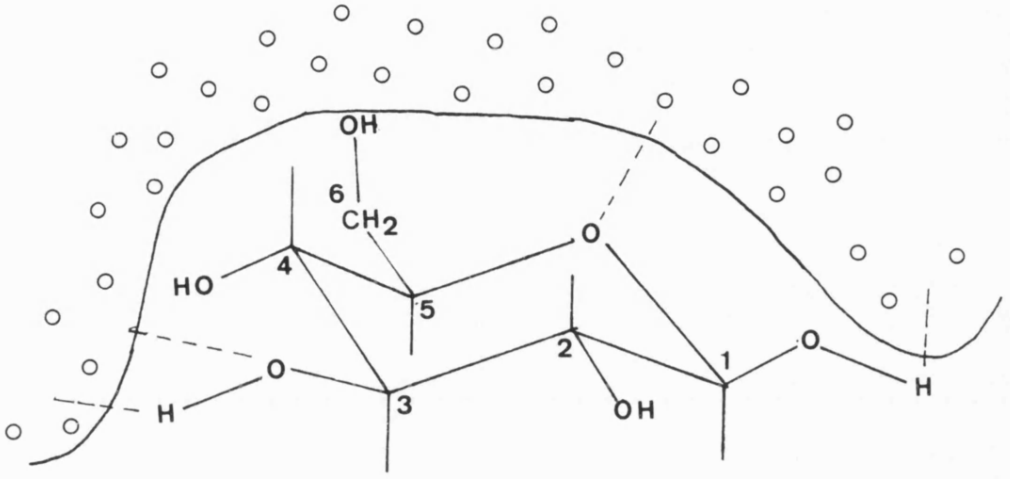
systems and therefore represents the only potential site for selective binding and possible transport of bulky substituents into the trypanosome. The proposed model (Figure 51) indicates a close approach to the binding site at the C-6 position. Evidence of lack of inhibition of C-6 analogues with bulky substitutions is also required to confirm the proposed model.

The inhibition experiments were carried out effectively under zero-trans conditions and thus measure inhibition of binding to the external site only. Side-specific analogues have been used in the adipocyte to demonstrate asymmetry in the inner and outer binding sites of the transport system which is not shown by the kinetics for a transported sugar in this system (Holman and Rees, 1982). Similar experiments would need to be carried out with trypanosomes to investigate whether the transporter is symmetrical with respect to both kinetics and specificity. Such data would be revealing in terms of the mechanism of the transport process in T. brucei.

5.4: The relationship between uptake and phosphorylation

When 1-deoxy-D-glucose was examined as a substrate for hexokinase from a crude trypanosomal extraction, the rate of 1-deoxy-D-glucose phosphorylation was less than 0.1% of the rate measured using D-glucose.

It was for this reason that 1-deoxy-D-glucose was considered as a non-metabolisable analogue. However



Solution

Figure 51: Model for the proposed structure of the D-glucose-carrier complex in T. brucei. Broken lines represent probable hydrogen bonds.

later studies using TLC analysis of transported 1-deoxy-D-glucose revealed that approximately 50% was in the phosphorylated form, indicating that in vivo 1-deoxy-D-glucose is a substrate for either hexokinase or another means of phosphorylation. It was at this stage that the presence of a group translocase system for sugar uptake was investigated. Such a system is attractive as it provides a means of regulation at the uptake stage and substrate is accumulated without expending metabolic energy (Postma and Roseman, 1976; Waygood et al., 1984).

The uptake experiments using 6-deoxy-D-glucose indicated that phosphorylation of the substrate was not necessary for transport to occur but this does not preclude close coupling of the transport and phosphorylation process. A linked transport and phosphorylation step has been proposed for the yeast D-glucose transport system (Van Steveninck and Rothstein, 1965; Jaspers and Van Steveninck, 1975; Tijssen et al., 1984). The demonstration of a similar V_{max} for sugar transport and for D-glucose metabolism in this thesis could also be regarded as being consistent with a linked system.

Bisson and Fraenkel, (1983) showed that in kinase deficient mutants of Saccharomyces cerevisiae, high affinity uptake of 6-deoxy-D-glucose was lost. This indicates that the kinase is necessary for high affinity transport to occur, but not by direct phosphorylation of the substrate as 6-deoxy-D-glucose cannot be

phosphorylated at the C-6 position. It is possible that 6-deoxy-D-glucose may still bind to the kinase during the transport process. The work with yeast shows that 6-deoxy-D-glucose can be transported by a linked transport phosphorylation process. However, this would require hexokinase to be present in the cytosol or associated with the membrane whereas hexokinase is reported to be located almost entirely within the glycosome (Misset and Opperdoes, 1984).

Studies on the extent of phosphorylation of 1-deoxy-D-glucose after long (20 minutes) preincubation without metabolisable substrate, indicated that approximately 50% of 1-deoxy-D-glucose was phosphorylated within a 3 second uptake period (See Figure 23). As these preincubation times were sufficient to stop cellular motility it was assumed that ATP levels had been completely depleted. However some supply of phosphate energy (ATP or ADP + myokinase activity) must remain for phosphorylation of transported 1-deoxy-D-glucose. One possibility is that ATP levels remain constant inside the glycosome and can be used to phosphorylate substrate once inside the organelle. There is no net change in ATP levels within the glycosome during glycolysis (Opperdoes, 1987). It is therefore possible that, if substrate levels are depleted, only the net ATP provided by the action of pyruvate kinase on phosphoenol pyruvate in the cytosol is depleted. As no substrate enters the glycosome, no ATP would be

consumed until substrate is restored ie, 1-deoxy-D-glucose which is then phosphorylated until all available ATP is utilised. Alternatively, if ATP levels do become depleted in the glycosome, uptake of substrates may activate myokinase, also present within the glycosome, to synthesise ATP from ADP.

It still remains to be established whether carrier-mediated transport occurs in the glycosome. Since functional glycosomes have never been produced the metabolic processes that occur within the glycosome cannot be fully understood. Recently, it has been reported that any small neutral and charged molecule can freely diffuse into and out of the glycosome (Patthey and Deshusses, 1987).

If this were the case it is difficult to see how the presence of the glycosome could confer significant advantage to the trypanosome. In particular, the proposed synthesis of net ATP by reversal of glycerol kinase during anaerobic respiration could not occur as high levels of α-glycerol phosphate are thought to be necessary for the reaction to proceed (Hammond and Bowman, 1980). It is possible that transport across the plasma membrane is not coupled to phosphorylation but transport across the glycosomal membrane is coupled to phosphorylation. However, it is most likely that phosphorylation of substrate within the glycosome relies on the high activity of hexokinase (Opperdoes and Borst, 1977).

Microbial transport is usually by means of high-affinity active uptake systems as substrates are often present at very low concentrations. There are however some microbial facilitative diffusion transport systems, the sugar transport system of Zymomonas mobilis for example (Dimarco and Romano, 1985). In this case, substrate levels are high and the energy yield of metabolism (via the Entner-Doudoroff pathway) is only 1 ATP molecule per molecule of D-glucose. This is a similar situation to the bloodstream form of T. brucei and is instructive in rationalising the evolution of a non energy-linked non-concentrative mode of uptake.

5.5: Future work

A) The specificity study could be extended to clarify the requirements at the C-6 and C-2 position. This would establish whether the model for the substrate-carrier complex proposed in this discussion is correct. Specificity studies on the inside binding site could provide evidence for the mechanism of the transport process.

B) Should further specificity studies confirm the unique spatial freedom at the C-2 position of the trypanosomal transporter binding site, the specific uptake of 'metabolic poisons', or the binding of high-affinity or irreversable inhibitors could be investigated with the aim of producing an anti-trypanosomal treatment. The potential effectiveness

of such inhibitors is enhanced by the accessibility of the transporter protein to suitable agents. Also, as transport does appear to be rate-limiting for metabolism, an inhibitor may slow the metabolism of trypanosomes sufficiently to affect the course of infection of the host.

C) The methodology described in this thesis allows the transport process to be studied in isolation from the metabolism that occurs within the cell. The transport site could therefore be investigated as a site of action for other trypanocidal agents. For example, preliminary studies on inhibition of uptake and efflux of 6-deoxy-D-glucose by trivalent arsenical compounds (results not shown) have indicated that the transport step may be an important site of action. Such studies could also reveal the amino acids present in the active site of the transporter.

D) The transport protocols could be adapted to study uptake and efflux of substrates into the glycosome. To establish whether carrier-mediated transport occurs in these organelles would significantly advance the understanding of the function of the glycosome.

5.6: Conclusions

Although the bloodstream form of T. brucei is only one stage of the life-cycle of one species of trypanosome, it is important in terms of the infection of mammals with African trypanosomiasis. Advances in

the treatment of the victims of Sleeping Sickness are most likely to result from a greater understanding of the potential sites for chemotherapeutic attack. In this context, the work in this thesis has attempted to improve the understanding of the hexose transport process in T. brucei and begin to examine the suitability of this process as a site for the action of trypanocidal agents.

Although much more work is required, the results of this study have shown that, like many other aspects of the biochemistry and morphology of T. brucei, the transport process differs in some respects from mammalian systems. The transport process may thus be suitable for the action of a selective trypanocidal agent, based on the differing specificities of the trypanosomal and mammalian transport of metabolic substrates.

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Sugar transport in *Trypanosoma brucei*: a suitable kinetic probe

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A transport assay has been developed for use in the investigation of 1-deoxy-D-glucose uptake in trypanosomes. 1-Deoxy-D-glucose has high affinity for the trypanosome sugar transport system (net influx $K_m = 4.03 \pm 0.42$ mM; $V = 0.052 \pm 0.005$ mM·s^{-1}. D-Glucose oxidation is competitively inhibited by 1-deoxy-D-glucose. However, we show that 1-deoxy-D-glucose is not a substrate for metabolism and that the competition occurs because of interaction at the transport system. D-Glucose competitively inhibits 1-deoxy-D-glucose influx.

(Trypanosoma brucei) *Sugar transport* *D-Glucose* *1,5-Anhydro-D-glucitol* *1-Deoxy-D-glucose*

1. INTRODUCTION

The long slender bloodstream form of African trypanosomes of the *brucei* group is totally dependent on glycolysis for ATP production, and consumes D-glucose at rates which are up to 10-times those of the most glycolytically active mammalian cells (review [1]). This high flux is favoured by the localisation of most of the glycolytic enzymes in the glycosome [2] and the possible further compartmentation of these enzymes in a multi-enzyme complex [3]. Glycolytic flux in *brucei* trypanosomes appears to be uncontrolled in the sense that little evidence has emerged for modulation at the enzyme level. Studies of the classical glycolytic pacemakers, hexokinase [4,5], pyruvate kinase [6] and phosphofructokinase [4,7] have shown that these enzymes are unlikely to play a regulatory role through interactions with metabolic intermediates acting as positive or negative effectors.

Although much work has been published on the nature and function of glycolysis in these organisms, the initial step, i.e. the transport of D-glucose across the plasma membrane, has received relatively little attention. Early studies of sugar transport in *Trypanosoma gambiense* [8,9] were interpreted as providing evidence for separate transporters for D-fructose and for D-glucose. More recently, Gruenberg et al. [10] have reported kinetic studies of D-glucose and 2-deoxy-D-glucose transport in *T. brucei* and concluded that a D-glucose transporter exists in this organism and that the uptake process is the rate-limiting step of D-glucose metabolism. However, as pointed out by Visser et al. [11] the high glycolytic flux poses a problem in the interpretation of uptake experiments involving metabolised sugars since most of the results published represent steady-state rather than initial rate measurements. It is clear that meaningful transport kinetics can only be obtained with intact cells by using D-glucose analogues which are non-metabolised and not phosphorylated. Such analogues would allow the dissection of effects of potential trypanocidal agents on transport from those on metabolism. We have found that 3-*O*-methyl-D-glucose, the analogue most often used for such studies in mam-

Dedicated to S.P. Datta upon his retirement as Managing Editor of FEBS Letters

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malian cells, is a very poor substrate for the trypanosome transporter and we have looked for a suitable alternative. We now report kinetics of uptake of 1,5-anhydro-D-glucitol and we show that this analogue is a good non-metabolised substrate for the trypanosome transporter.

2. EXPERIMENTAL

2.1. *Materials*

Enzymes and cofactors were purchased from Sigma. 1,5-Anhydro-D-glucitol (1-deoxy-D-glucose) was synthesised as described by Barnett et al. [12]. Radiolabelled 1-deoxy-D-glucose was prepared similarly except that D-[U-^{14}C]glucose (275 mCi/mmol) was used. All other reagents were of analytical grade.

Cells of the long slender form of *T. brucei* were isolated from Wistar or Sprague-Dawley rats (250–400 g) infected with $1–3 \times 10^7$ cells of strain MITat 1.1 as described [13], and purified on a DEAE-cellulose column [14]. After preparation the cells were kept at 0°C in storage buffer (98 mM NaCl, 22 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM KCl; pH 8.0) containing 10 mM D-glucose.

2.2. *Transport measurements*

Immediately before the uptake experiment D-glucose was removed by centrifuging a cell suspension, suspending the pellet in cold storage buffer, recentrifuging and resuspending the pellet in an appropriate volume of buffer for the transport experiment. All centrifugations were carried out in an Ole-Dich refrigerated microfuge (15000 × *g*, 20 s, 0°C) to ensure rapid processing of the cells.

The cells were preincubated at 37°C for 30 s and then 0.1–0.3 μCi 1-deoxy-D-[U-^{14}C]glucose, diluted with an appropriate concentration of unlabelled sugar was added. At appropriate intervals duplicate 200-μl samples were removed, using a multi-channel pipette, and dispersed into vials containing 1 ml ice-cold storage buffer. The vials were immediately centrifuged and the supernatants removed. The pellets were resuspended and the wash stage was repeated; the supernatants were carefully removed. Carry-over of radiolabel or zero time uptake was estimated from samples which were added directly to ice-cold buffer. The cell pellets were lysed in distilled water and dispersed into scintillant for liquid scintillation counting. For the estimation of K_m generally just a single time point was used (30 s). In addition, for the lowest substrate concentration used, samples were taken after cellular equilibration with substrate had been reached (2–3 min). This provided an estimate of the volume of the cells accessible to the substrate so that rates could be calculated in units of concentration·time^{-1}. For the D-glucose inhibition experiment, cells were suspended in buffer containing the appropriate concentration of D-glucose before the addition of 1-deoxy-D-glucose. Kinetic constants were calculated using the direct linear plot [15] or by least squares.

2.3. *Hexokinase assay*

Hexokinase activity was determined by measuring the production of ADP in a coupled system containing pyruvate kinase and lactate dehydrogenase at 25°C. The assay buffer (pH 7.4) contained 0.4 M triethanolamine, 2 mM EDTA, 4 mM $MgCl_2$, 4 mM KCl, 0.5 mM ATP, 0.5 mM phospho*enol*pyruvate, 0.5 mM NADH, 5 units pyruvate kinase, 5 units lactate dehydrogenase. Hexokinase was assayed in a sonicated cell lysate or a disrupted glycosomal preparation prepared essentially as described by Oduro et al. [16]. A correction was made for NADH oxidase activity. NADH oxidase activity was determined by omission of ATP and phospho*enol*pyruvate and was found to be less than 0.5% of maximal hexokinase activity. Trypanosome respiration rates were estimated at 37°C using a Clark oxygen electrode.

3. RESULTS AND DISCUSSION

1-Deoxy-D-glucose (10 mM) showed no detectable ADP production in an assay for hexokinase activity (<0.1% of the rate with D-glucose under assay conditions) in either sonicated cell lysates or in a disrupted glycosome preparation. 1-Deoxy-D-glucose, at concentrations up to 10 mM, did not inhibit hexokinase activity, even at the lowest D-glucose concentration tested (0.25 mM).

Oxidation of D-glucose in trypanosomes is however inhibited by 1-deoxy-D-glucose. The inhibition was substrate-antagonised as shown in fig.1. Here the fractional inhibition at fixed inhibitor concentration decreases with increasing D-

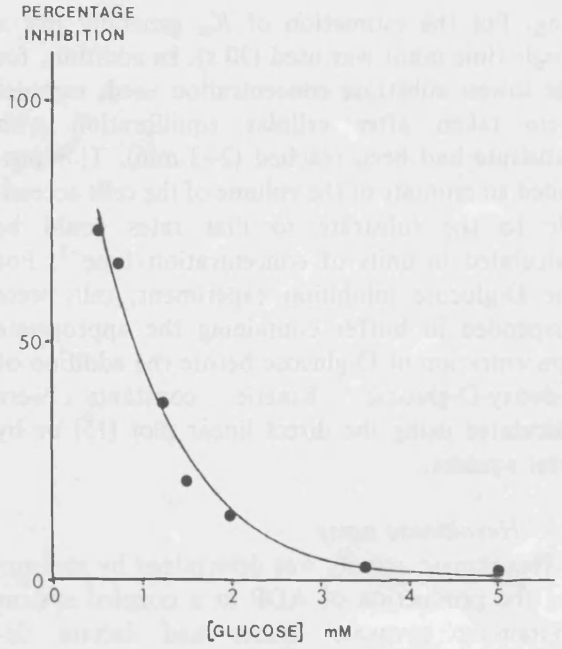

Fig.1. Inhibition of rate of D-glucose respiration in *T. brucei* by 40 mM 1-deoxy-D-glucose. Percentage inhibition is given by $100 \times (1 - (v_i/v_o))$ where v_i and v_o are the rates of oxygen utilisation in the presence and absence, respectively, of 1-deoxy-D-glucose.

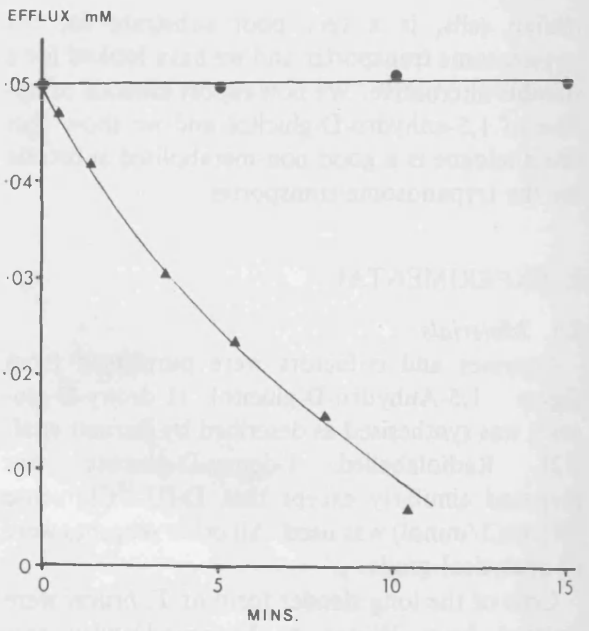

Fig.2. Efflux of 1-deoxy-D-glucose from *T. brucei* (equilibrated with 50 μM 1-deoxy-D-glucose) by dilution with 5 vols buffer at 0°C (●) and 37°C (▲). For details see text.

glucose concentration. Because of the lack of interaction of D-glucose and 1-deoxy-D-glucose at the hexokinase level the observed effect of 1-deoxy-D-glucose on respiration is likely to be mediated at the membrane transporter.

In order to set up procedures for investigating sugar interactions with their transport system a number of agents were tested as stoppers in uptake experiments. At 37°C neither *p*-hydroxymercuribenzoate, nor mercuric chloride, nor cytochalasin B was totally effective. However, a 5-fold dilution with ice-cold buffer was sufficient to prevent efflux of label. In the experiment shown in fig.2 cells were equilibrated with 50 μM 1-deoxy-D-glucose and then diluted into ice-cold buffer. No efflux was observed over a time course which was much longer than the cell processing time required for estimating trapped label (see section 2).

The time course for 50 μM 1-deoxy-D-glucose uptake is shown in fig.3. From the equilibrium level of substrate it is calculated that the available cell volume is 5.9 μl/10^8 cells. This value is midway between those reported by other workers [17,18]. The approach to equilibrium shown is typical for the flux of a substrate concentration below the K_m. The data fitted the integrated rate equation [19] from which an initial rate was calculated (fig.3, inset). This showed that a 30 s time point gave an accurate estimate of the initial rate. In experiments in which K_m estimates for 1-deoxy-D-glucose were made a 30 s time point was generally used. A 45 s time point gave similar results. Variation of initial rates with 1-deoxy-D-glucose concentrations followed Michaelis-Menten kinetics. From the direct linear plot shown in fig.4 $K_m = 4.03 \pm 0.42$ mM and $V = 0.052 \pm 0.005$ mM·s^{-1} were calculated. The clustering of the intersections of this plot gives a visual estimate of the error in the kinetic parameters. Uptake rates were competitively inhibited in the presence of added D-glucose (fig.4, inset). In these experiments the conditions were adjusted to ensure that the D-glucose concentration outside the cells did not change by more than 10% during the incubation. The estimated K_i is 0.33 ± 0.05 mM but since the D-glucose concentration inside the cells is unknown the extent to which this value represents a true transport inhibition constant is unclear. However, this operational

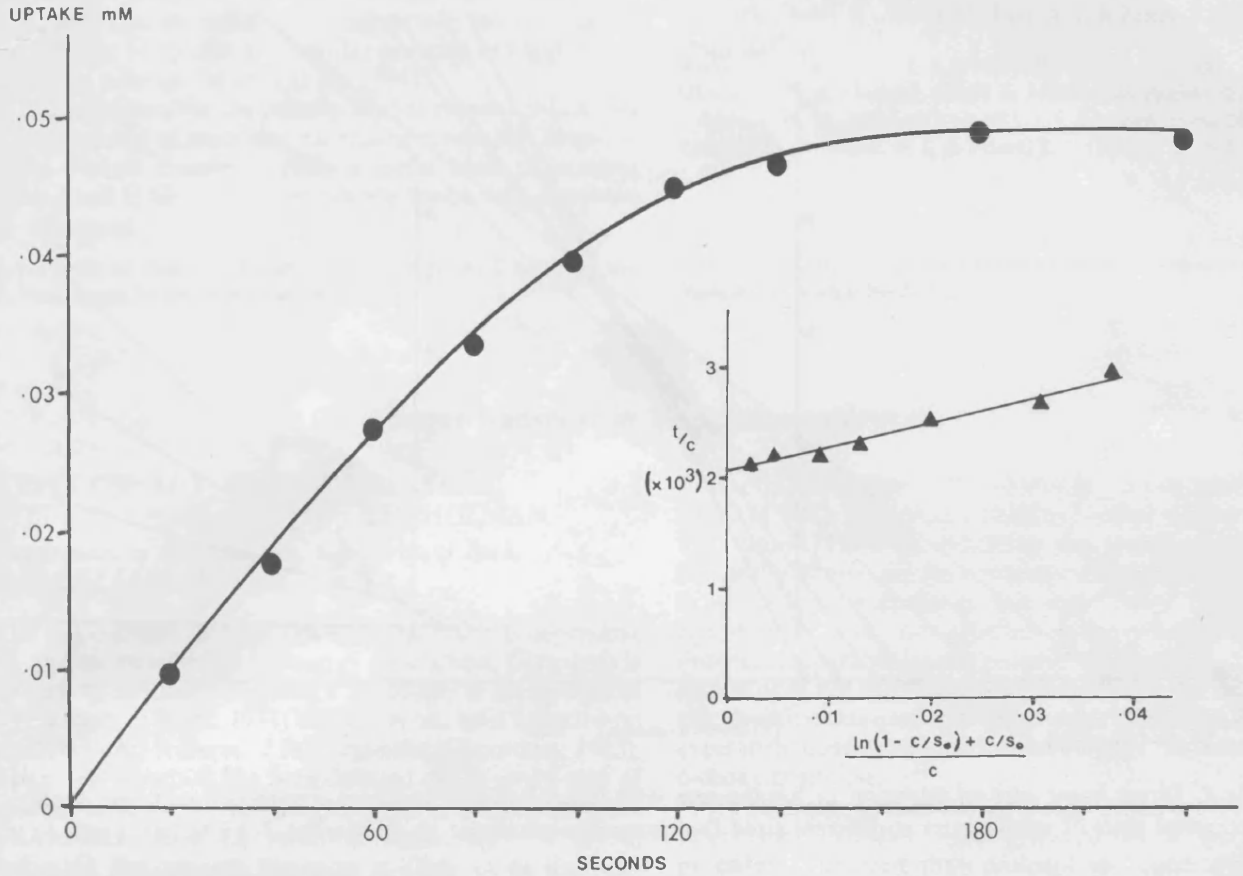

Fig.3. Time course of 1-deoxy-D-glucose uptake (50 μM) by *T. brucei* at 37°C. Inset: data fitted to integrated rate equation [19].

K_i is 10-fold lower than the half-maximal saturation constant for D-glucose oxidation and 3-fold lower than the K_i for D-glucose inhibition of 2-deoxy-D-glucose uptake and than the K_m for D-glucose uptake [10].

Further investigations of the specificity of this transport system are required. However, it is clear that this system is dissimilar to both bacterial and mammalian transport systems for sugars. *E. coli* GalP and MG phosphotransferase systems do not transport 1-deoxy-D-glucose [20] (P.J.F. Henderson, personal communication). Mammalian sugar transport systems will transport 1-deoxy-D-glucose but removal of the hydroxyl at carbon 1 results in a large affinity loss. The K_m in mammalian systems is 80–90 mM [12,21] which is a considerably lower affinity than that which we now report for the trypanosome system. Another marked difference between the trypanosome and mammalian systems is the difference in affinity for 3-*O*-methyl-D-glucose. In the mammalian system the addition of the methyl group at C-3 has no effect on affinity but in trypanosomes a huge loss of affinity occurs. The rates of 3-*O*-methyl-D-glucose uptake in trypanosomes are less than 10% of the rates of 1-deoxy-D-glucose uptake. However, 6-deoxy-D-glucose (quinovose) was found to be an effective inhibitor of 1-deoxy-D-glucose uptake, and this analogue may also be suitable for studies of sugar transport in these cells.

The influx V for trypanosome sugar transport is slower than that for mammalian systems. In view of the much higher glycolytic rate in trypanosomes it seems likely that in these organisms overall D-glucose oxidation rates are more rate limited by membrane transport than in mammalian cells.

ACKNOWLEDGEMENT

We are grateful to the Wellcome Trust for financial support.

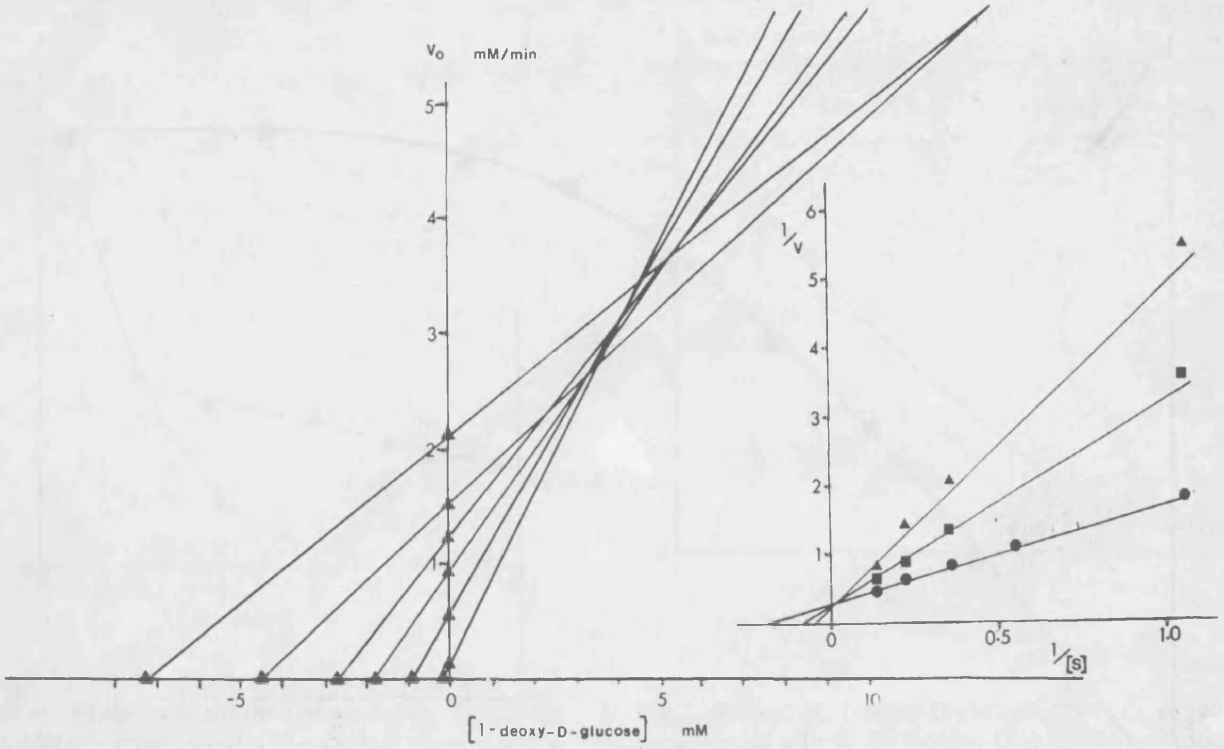

Fig.4. Direct linear plot of variation of uptake rate with initial 1-deoxy-D-glucose concentration. Inset: double-reciprocal plots of uptake rates at different fixed D-glucose concentrations: no glucose (●), 0.5 mM (■), 1 mM (▲).

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deposition. Our failure to demonstrate any reduction in the amount of mixed muscle protein does not preclude the possibility that chronic ethanol ingestion in rats may reduce the amount of specific myofibrillar proteins as suggested in alcoholic patients (Martin *et al.*, 1984).

In conclusion, the chronically treated ethanol-fed rat is a suitable model to study skeletal muscle myopathy. Anatomically distinct muscles provide a useful basis to examine types I and II fibres and demonstrate contrasting responses to treatment.

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Sugar transport in *Trypanosoma brucei*

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The bloodstream form of *Trypanosoma brucei* is dependent on glucose metabolism for energy production. Glycolysis is thought to be localized mainly if not totally in the glycosome (Opperdoes & Borst, 1977) and much has been reported on the functional features of this organelle (Opperdoes, 1985). Much less attention has been directed at the initial step of glucose metabolism, i.e. the transport of sugar across the plasma membrane. The results of Gruenberg *et al.* (1978) indicated that glucose transport is likely to be the rate-limiting step in glucose metabolism, but those studies involved the use of 2-deoxyglucose which is known to be a substrate for hexokinase. Recently Game *et al.* (1986) reported that 1-deoxy-D-glucose may be a suitable analogue for studying sugar transport in *T. brucei*. Their results on 1-deoxy-D-glucose influx demonstrated that the V for trypanosome sugar transport is slower than that for mammalian systems. However, attempts to characterize efflux and exchange kinetics of 1-deoxy-D-glucose effect were frustrated by a slow but significant phosphorylation of the analogue.

Game *et al.* (1986) also reported that 6-deoxy-D-glucose was an effective inhibitor of 1-deoxy-D-glucose uptake. As this analogue is unlikely to undergo any metabolism, we have investigated the kinetics of its uptake and efflux in intact cells and have also studied D-glucose transport in vesicles prepared from trypanosome plasma membranes.

6-Deoxy-D-glucose was synthesized from 6-chloro-α-methyl-D-glucoside (Evans & Parrish, 1972) which was converted to the 6-iodo compound and then catalytically reduced with tritium gas (Amersham). This tritiated product was hydrolysed to remove the glycosidic methyl group and the resultant [6-^{3}H]deoxy-D-glucose was then purified by paper chromatography.

Kinetic parameters for 6-deoxy-D-glucose were determined at 20°C. Net influx and efflux showed similar K_m and V_{max} values (Table 1) indicating the transport system is kinetically symmetric. An equilibrium exchange experiment in which isotope exchange flux was studied in cells pre-equilibrated with non-labelled 6-deoxy-D-glucose also showed comparable kinetic parameters (Table 1). Thus the system does not show accelerated exchange. An integrated rate equation was used to determine the infinite-*cis* K_m. This experiment confirmed kinetic symmetry for the transport of 6-deoxy-D-glucose.

We have also studied hexose transport in purified plasma membranes. Vesicles were prepared by a freeze-thaw procedure. The freeze-thaw protocol was found to be essential to remove cytoskeletal components associated with the plasma membrane sheets. We have found that an infinite-*trans* protocol is most useful for studying the kinetics of transport in this isolated membrane system. In this procedure vesicles are loaded with 100 mM-D-glucose and then diluted into a buffer containing radiolabelled D-glucose at a range of concentrations. The high concentration of D-glucose inside traps (by counterflow) the radiolabelled D-glucose as it enters the vesicles. The estimated K_m is shown in Table 1. The K_m of 14 mM is considerably higher than the affinity constant estimated by using intact cells and further experiments will investigate the reasons for the difference.

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Table 1. *Kinetic constants for sugar transport in T. brucei*

Substrate	Procedure	K_m (mM)	V_{max} (mM s^{-1})
6-Deoxy-D-glucose	Zero-*trans* entry	2.17 ±0.068	0.279 ±0.006
6-Deoxy-D-glucose	Zero-*trans* exit	6.063 ± 1.227	0.863 ± 0.109
6-Deoxy-D-glucose	Infinite-*cis* entry	6.212 ± 1.197	0.601 ± 0.052
6-Deoxy-D-glucose	Equilibrium exchange	3.0 ± 0.332	0.965 ± 0.067
D-Glucose	Infinite-*trans* entry in vesicles	14.33 ± 2.55	–

Proteolytic degradation of mucus in the colon

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A continuous adherent layer of insoluble mucus gel is found on the colonic mucosal surface, mean thickness 150 ± 110 μm in the rat (Sakata & Engelhardt, 1981). Such a mucuos layer will provide a protective barrier against harmful agents in the lumen and mechanical damage from motile forces of digestion. The protective gel layer is formed by polymeric glycoproteins which are fragmented by proteases to dissolve the mucous layer (mucolysis) (Allen *et al.*, 1984; Bell *et al.*, 1985). Recent studies of proteolytic activity in stools (using non-mucous protein substrates) showed that inflammatory bowel disease patients had significantly higher levels of activity compared to non-symptomatic controls (Corfield *et al.*, 1986). If such proteolytic activity reflects excessive mucolytic activity (mucous degrading) then this may be an important factor in disruption of the colonic mucosal barrier in inflammatory bowel disease. Here we demonstrate mucolytic activity in human faecal extracts from non-inflammatory bowel disease patients.

Glycoproteins from pig and human colonic mucous and pig gastric mucuous were extracted in proteinase inhibitors [1.0 mM-phenylmethylsulphonyl fluoride (PMSF), 50 mM-iodoacetamide, 100 mM-α-aminohexanoic acid, 5 mM-benzamidine HCl, 1.0 mg l^{-1} soybean trypsin inhibitor and 10 mM-Na_2 EDTA in 0.5 M-Tris/HCl pH 8.0] and purified by two successive equilibrium centrifugation steps in 3.5 M-$CaCl_2$. Purified glycoproteins were largely excluded (> 60%) from Sepharose CL-2B and were fragmented into smaller species, included on Sepharose CL-2B, by reduction (0.2 M-mercaptoethanol 48 h, 4°C) and exhaustive proteolysis [1 : 100 (w/w) papain : glycoprotein, 48 h, 60°C]. This fragmentation was reflected by a substantial drop in solution viscosity (approximately 10-fold on papain digestion of pig colonic mucous glycoprotein at 4 mg ml^{-1}).

Human faecal extracts (from non-inflammatory bowel disease patients) were obtained by suspending samples of stool in 4 vol. of M/15 M phosphate buffer pH 7.5, containing 50 mM-NaCl and centrifuging at 10 000 ***g*** for 15 min at 4°C. Protease activity was measured by a sensitive trinitrobenzene sulphonic acid assay for formation of new peptide *N*-terminals at pH 7.5 and 37°C using succinyl albumin or purified mucous glycoprotein as substrate (Hutton *et al.*, 1986). Protolytic action on succinyl albumin by the extracts was inhibited by soybean trypsin inhibitor and PMSF (1 mM) [but not by iodoacetamide (50 mM) or Na_2 EDTA (10 mM)] suggesting that the activity was due to a trypsin-like serine-dependent protease. Subsequently, levels of faecal protease activity (against succinyl albumin) were quantified as equivalents of porcine pancreatic trypsin (Sigma) activity by weight. On this basis, faecal protease activity was completely inhibited by soybean trypsin inhibitor [1 : 1 (w/w) inhibitor : faecal protease activity] and the synthetic polyacrylate Carbomer 934P [1000 : 1 (w/w) polyacrylate : faecal protease activity]. PMSF [100 : 1 (w/w)] inhibited activity by ~60%. Mucolytic activity of the faecal extracts was assayed by monitoring the fall in specific viscosity of solutions of purified mucous glycoproteins, as a function of time, on incubation with faecal extract at pH 7.5 and 37°C. Mucolysis was also followed by measuring the increase in free *N*-terminals and the amount of glycoprotein included in Sepharose CL-2B. In this manner, extracts were shown to proteolytically digest purified pig gastric and colonic mucous glycoproteins. For example, incubation of purified pig colonic muous glycoprotein with extract [0.1% enzyme : glycoprotein (w/w)] induced a rapid fall in the specific viscosity of the glycoprotein in the first 15 min of digestion (74.5% of the fall in specific viscosity after complete digestion) followed by a slower fall over the subsequent 48 h. This fall in viscosity was accompanied by an increase in the proportion of glycoprotein included on Sepharose CL-2B this being 35% at time 0, and 77% and 89% after 15 min and 24 h, respectively. Decrease in viscosity was also mirrored by an increase in the number of free *N*-terminals and a 50% drop in specific viscosity corresponded to 3.1×10^{-5} mol of peptide bonds cleaved. The synthetic polyacrylate Carbomer 934P [1200 : 1 (w/w) polyacrylate : faecal protease activity] inhibited mucolysis by 45%.

The polyacrylate Carbomer 934P, in addition to inhibiting degradation of mucus, was shown to have a potential role in strengthening the protective mucous barrier. Carbomer 934P synergistically increased the viscosity of purified undegraded colonic mucous glycoproteins from both man and pig. Thus, viscosity of glycoprotein/carbomer mixtures (GC) was substantially greater than the sum of the individual viscosities (G+C). This synergistic increase in viscosity was approximately 460% at concentrations of glycoprotein and carbomer of 4 mg ml^{-1} and 2 mg ml^{-1}, respectively.

These studies demonstrate endogenous mucolytic activity, by proteases, exists in the lumen of the colon *in vivo*. Such mucolysis may be an important factor in disruption of the colonic mucous barrier in disease.

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Abbreviation used: PMSF, phenylmethyl sulphonyl fluoride.

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